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The Neuroprotective and Therapeutic Effects of Anthocyanins and Their Metabolites In Vitro and in a Mouse Model of Amyotrophic Lateral Sclerosis

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THE NEUROPROTECTIVE AND THERAPEUTIC EFFECTS OF ANTHOCYANINS AND THEIR METABOLITES *IN VITRO* AND IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT

Anthocyanins, a unique class of flavonoid compounds, have recently come to the forefront of investigative research aimed at evaluating the potential applications of natural products to human health. Evidence demonstrating the beneficial effects of anthocyanin consumption has been reported for a myriad of conditions including cancer, cardiovascular disease, and lately, neurodegenerative disease. Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS) are characterized by the death of specific neuronal populations within the brain and spinal cord, leading to cognitive and/or motor impairment. While the etiology of many of these diseases is largely unknown, several factors have emerged as contributors to the neurodegenerative process. These include conditions such as oxidative and nitrosative stress, glutamate excitotoxicity, endoplasmic reticulum stress, protein aggregation, and neuroinflammation, which ultimately culminate in the death of susceptible neuronal populations. Since anthocyanins have been shown to modulate these phenomena in numerous ways, interest in evaluating their use as therapeutic agents for neurodegenerative diseases has grown. Additionally, the observation that the bioavailability of anthocyanins is very low following consumption relative to that of their metabolites suggests that anthocyanin metabolites may also play a significant role of mediating the beneficial effects of anthocyanin-rich diets. Therefore, we examined the neuroprotective and therapeutic effects of both anthocyanins and phenolic acid

metabolites derived from anthocyanins *in vitro* and in a mouse model of ALS. The results of our work reveal that different anthocyanin species possess differential neuroprotective effects *in vitro* against toxicity induced by nitric oxide and define a novel neuroprotective mechanism for cyanidin-*O*-3-glucoside under these conditions. Furthermore, we demonstrate that presymptomatic supplementation with an anthocyanin-enriched extract from strawberries significantly delays disease onset and extends survival in the transgenic G93A mutant Cu, Zn-superoxide dismutase ($hSOD1^{G93A}$) mouse model of ALS. These observations correlate with significant preservation of hind limb grip strength and function in mice treated with anthocyanins. Anthocyanin supplementation is also shown to improve histopathological indices of disease, reducing reactive astrogliosis in lumbar spinal cord tissue, and preserving neuromuscular junctions in gastrocnemius muscle tissue. We next illustrate that phenolic acid metabolites derived from anthocyanins display distinct and complimentary neuroprotective effects *in vitro* against a diverse array of stressors in cerebellar granule neurons. Lastly, our work shows that treatment with protocatechuic acid, a metabolite of cyanidin-*O*-3-glucoside, beginning at disease onset provides significant therapeutic benefit to $hSOD1^{G93A}$ mice, extending survival and preserving hind limb grip strength in animals supplemented with this compound. The results of this dissertation are the first to evaluate the therapeutic efficacy of anthocyanins and their metabolites for the treatment of ALS. Collectively, these data demonstrate that both anthocyanins and their metabolites may be of significant clinical benefit for treating this insidious disease, and suggest that further preclinical and clinical examination of these compounds is warranted.

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CHAPTER ONE: INTRODUCTION

With the advent of rapid medical advance, populations around the world have experienced a steady and substantial increase in average life expectancy, and with it, a tremendous increase in the incidence of neurodegenerative disease. Cases of Alzheimer's disease and Parkinson's disease are by far the most prevalent among this increase; however recent evidence suggests that less common diseases, such as amyotrophic lateral sclerosis (ALS), the focus of this dissertation, may also be on the rise (Ingre et al. 2015).

Neurodegenerative diseases are characterized by death of specific neuronal populations in the brain, brain stem and spinal cord, leading to significant impairments in cognitive and/or motor performance, and eventual death. ALS, in particular, is marked by the death of both upper and lower motor neurons, which causes severe muscle wasting and atrophy, paralysis, and death, usually due to respiratory failure. Two forms of ALS are currently recognized: familial forms of the disease, which are caused by inherited genetic factors, and sporadic forms of the disease for which the etiology is unknown. Of these, sporadic forms of ALS account for the vast majority of all cases, comprising 90% of the patient population. Both forms of ALS progress rapidly, and most patients will succumb to this insidious disease within 2-5 years of diagnosis, a grim prognosis that has not changed in the 75 years since the disease was made famous by Lou Gehrig. Indeed, the development of therapeutic agents for ALS has been extremely limited, and only one FDA approved drug, Riluzole, exists to treat both sporadic and familial forms of disease.

Riluzole, an anti-glutamatergic compound, modestly extends the lifespan of ALS patients by 2-3 months, but has no reported benefit on quality of life (Miller et al. 2012). Furthermore, due to a host of side effects, many patients choose to forego treatment with this drug entirely and instead seek alternative, often unproven, treatments for their disease in the form of dietary supplements. Therefore, it is imperative to identify new therapeutic agents that can be used to safely and effectively treat ALS.

The primary focus of this dissertation is to examine a class of potential therapeutic agents known as anthocyanins for their ability to attenuate multiple facets of ALS disease pathology while also defining their mechanisms of action. Additionally, the therapeutic benefit and effects of these unique compounds on disease progression and pathology is explored *in vivo* in a mouse model of ALS.

1.1 The Pathological Basis of Motor Neuron Death in ALS

Until recently, the complex nature of ALS was not fully appreciated, making this disorder very difficult to treat effectively. However, the multi-faceted nature of neurodegeneration has recently become a topic of intense scrutiny in the effort to identify new therapeutic targets for this disease. Several common mechanisms are now believed to underlie motor neuron death in both sporadic and familial forms of ALS, including oxidative stress, excitotoxicity, neuroinflammation, dysregulation of protein homeostasis, and apoptotic cell death. These factors are discussed below, and summarized in Figure 1.1.

1.1.1 The Role of Oxidative Damage in ALS

Oxidative damage is one of the most common features among diverse neurodegenerative diseases, including ALS, which has made it an appealing therapeutic target. This type of damage occurs when the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the cell overwhelms endogenous antioxidant defenses, resulting in oxidative and nitrosative stress, respectively. Both forms of cellular stress lead to severe oxidative damage of vital cellular macromolecules such as lipid membranes, proteins, and DNA, which culminates in the induction of neuronal cell death (Lin and Beal, 2006). In neurons, the primary sources of oxidative stress are dysfunctional mitochondria, commonly thought of as the "powerhouses" of the cell, responsible for producing the majority of cellular energy, and functional loss of endogenous antioxidant defenses normally involved in the detoxification of ROS and RNS.

Mutations in Cu, Zn-superoxide dismutase (SOD1) are associated with familial forms of ALS, with over 150 mutations identified in this gene to date, accounting for approximately 20% of all familial cases of this disease (Rosen et al. 1993). To date, models utilizing mutant forms of SOD1 are by far the best studied, and a majority of what is currently known about the role of oxidative stress in ALS stems from the use of these models.

On their own, mutations in the SOD1 protein can contribute to the induction of oxidative stress in that they may induce toxic gain-of-function activities, which enhance the production of ROS within the cell (Yim et al. 1996). However, recent evidence has indicated that one of the predominate ways by which some forms of mutant SOD1

contribute to the induction of oxidative stress within the cell is through selective association with mitochondrial membranes, which causes significant mitochondrial damage and impaired energy metabolism (Ferri et al. 2006). This in turn causes mitochondria to produce greater levels of ROS, which cannot be readily detoxified by the cell.

Mitochondrial SOD1 aggregates have also been shown to include Bcl-2, a crucial pro-survival protein involved in maintaining levels of glutathione (GSH) within mitochondria (Pasinelli et al. 2004, Wilkins et al. 2012). This essential antioxidant is responsible for scavenging ROS generated by mitochondria through the process oxidative phosphorylation in the electron transport chain, as well as maintaining redox homeostasis in many other parts of the cell (reviewed by Aoyama and Nakaki, 2015). It is noteworthy then, that GSH depletion has been documented in spinal cord mitochondria isolated from mice harboring a human mutant SOD1 transgene (Chi et al. 2007, Pesaresi et al. 2011). These mice display a significant decrease in the ratio of reduced to oxidized GSH in spinal cord mitochondria, indicative of either a decrease in the reduced form of GSH, and/or an increase in levels of oxidized glutathione (GSSG), both of which could be indicative of a highly oxidizing cellular environment (Pesaresi et al. 2011). Moreover, GSH depletion appears to be correlated with disease progression, indicating that the loss of antioxidant capacity within affected neuronal populations may play an important role in the progression of the disease over time (Babu et al. 2008). This is supported by studies in mice expressing the human transgene for a mutant form of SOD1 harboring a glycine to alanine mutation at position 93 ($mSOD1^{G93A}$) in which these mice were crossed with mice heterozygous for deletion of the *SOD2* gene, which codes for the

mitochondrial specific Mn-superoxide dismutase (SOD2) protein. Transgenic mice that were also heterozygous for the *SOD2* deletion demonstrated a striking shift in disease progression, displaying both a significant increase in motor neuron pathology and shortened lifespan (Andreassen et al. 2000). Similarly, knockout of the regulatory subunit of γ-glutamylcysteine ligase, the rate-limiting enzyme responsible for GSH synthesis, in transgenic mSOD1^{G93A} mice accelerated motor neuron disease (Vargas et al. 2011). Collectively, these data highlight the role of oxidative damage in ALS pathogenesis, particularly as a result of mitochondrially derived ROS, and loss of endogenous antioxidant defenses.

While compelling, evidence for the role of oxidative damage in SOD1 linked familial ALS represents only about 2% of all ALS cases; however recent exploration of this topic has revealed that oxidative and nitrosative stress are also involved in other familial forms of the disease, and in sporadic ALS. Mutations in TAR DNA-binding protein-43 (TDP-43) are associated with some familial and sporadic forms of ALS, and TDP-43 aggregation in motor neurons is a hallmark of non-SOD1 familial ALS and sporadic ALS (Arai et al. 2006, Neumann et al. 2006). Interestingly, aggregation of TDP-43 can be recapitulated in neuronal cell lines through depletion of GSH, suggesting that GSH depletion caused by oxidative stress in ALS may be responsible in part for aggregate formation over the course of the disease (Iguchi et al. 2012). Furthermore, TDP-43 overexpression in *Drosophila* motor neurons enhances indices of oxidative stress such as protein carbonylation and elevated levels of antioxidant response enzymes (Zhan et al. 2015). Similar findings have also been observed in induced pluripotent stem cells derived from ALS patients harboring expansions in the *C9orf72* gene, a newly discovered

mutation believed to account for as much as 50% of all familial ALS cases (DeJesus-Hernandez et al. 2011, Renton et al. 2011, Gijselinck et al. 2012). These cells display increased levels of the antioxidant enzyme, catalase, suggestive of enhanced ROS production (Kiskinis et al. 2014).

Lastly, it is important to note that biomarkers of oxidative stress have been reported consistently in patients with sporadic ALS, including elevated levels of oxidized DNA and lipid peroxidation (Bogdanov et al. 2000, Simpson et al. 2004). Additionally, GSH depletion has been reported in erythrocytes and motor cortex from ALS patients, indicative of systemic reductions in antioxidant defenses, which are thought to contribute to oxidative stress in this disease (Babu et al. 2008, Weiduschat et al. 2014). Interestingly, there is also evidence emerging that wild type SOD1 may play a role in sporadic ALS as oxidized forms of this protein found in lymphocytes from ALS patients behave in a manner that is similar to their mutant SOD1 counterparts, forming mitochondrial protein aggregates with Bcl-2 (Guareschi et al. 2012). S-nitrosylation of protein disulfide isomerase (PDI), which is involved in protein folding and homeostasis, a process that is significantly disrupted in ALS, has also been identified, further illustrating that oxidative modification to important cellular components may contribute to ALS pathogenesis (Walker et al. 2010).

1.1.2 The Role of Excitotoxicity in ALS

Excitotoxicity is a phenomenon unique to neurons in which excitatory stimuli cause neurons to experience massive calcium influx, membrane depolarization, and subsequent death through production of ROS and RNS as well as activation of calcium-

dependent pro-death factors, such as calpains (Van Damme et al. 2005). Many factors are posited to contribute to this process in ALS, including disturbances in excitatory signaling by neurotransmitters, increased sensitivity of post-synaptic neurons to excitatory stimuli, and reductions in inhibitory signaling by interneurons (reviewed in King et al. 2016). While there is some debate about which of these factors is predominately responsible for inducing excitotoxicity in motor neurons, there is little doubt as to the involvement of excitotoxicity in ALS disease pathogenesis. This is most convincingly evidenced by the fact that to date, the only drug to have shown even modest efficacy for the treatment of ALS, Riluzole, is an anti-glutamatergic compound, which is thought to act by diminishing aberrant excitatory signaling in motor neurons (Miller et al. 2012).

Glutamate is the primary excitatory neurotransmitter in the central nervous system, and evidence for its dysregulation in ALS is abundant. Early reports suggest that glutamate levels are elevated in postmortem tissue and cerebrospinal fluid isolated from ALS patients, though recent work has brought these findings into question (Shaw et al. 1995, Spreux-Varoquaux et al. 2002, Wuolikainen et al. 2011). Nevertheless, emerging data have implicated enhanced glutamate release and increased neuronal firing as a potential sources of glutamate-induced excitotoxicity. An increased incidence of calcium-dependent spontaneous neurotransmitter release has recently been linked to endoplasmic reticulum (ER) stress, a condition that is garnering significant attention as a major factor in ALS (Nosyreva and Kavalali, 2010). It is believed that this spontaneous neurotransmission is related to enhanced sensitivity of synaptic vesicle release machinery to calcium signaling during activity-dependent depolarization of the cell. While this study

did not examine ALS specifically, it suggests that augmented neuronal activity, and specifically the release of neurotransmitters such as glutamate, may occur in response to ER stress, which could result in the induction of excitotoxicity in post-synaptic cells. This assertion is supported by recent data demonstrating that synaptosomes isolated from mice expressing the mSOD1^{G93A} human transgene demonstrate heightened glutamate release in response to neuronal depolarization and stimulation of metabotropic glutamate receptors by receptor agonists (Milanese et al. 2011, Giribaldi et al. 2013). Furthermore, reductions in glutamate loading into synaptic vesicles and a consequent decrease in glutamate release from pre-synaptic terminals preserves motor neuron viability in a mouse model of ALS, although this preservation does not correlate with improvements in disease progression and survival (Wootz et al. 2010).

In addition to evidence suggesting an increase in synaptic glutamate release, recent data also demonstrate that the ability of cells in the central nervous system (CNS) to clear glutamate from the synaptic cleft after its release is impaired in ALS. Under normal conditions, glutamate is rapidly removed from the synaptic cleft via reuptake by both neurons and neighboring astrocytes, glial cells that provide supportive functions to neurons in the CNS. This occurs predominately through the excitatory amino acid transporter (EAAT2) expressed by astrocytes; however it has been observed that EAAT2 expression is significantly reduced in spinal cord of both mice and rats carrying a mutant SOD1 transgene, as well as in tissue isolated from both familial and sporadic ALS patients (Rothstein et al. 1995, Fray et al. 1998, Sasaki et al. 2000, Allaman et al. 2011). Moreover, overexpression of EAAT2 in an ALS mouse model significantly delays disease onset, although no effect on survival was observed, which may suggest that

aberrant glutamate signaling plays a role in the early stages of the disease (Guo et al. 2003).

It has also been shown that motor neurons in ALS display increased sensitivity to glutamate excitotoxicity as a result of multiple factors. Expression levels of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, one of several types of ionotropic glutamate receptor, are intrinsically high in motor neurons (Van Den Bosch et al. 2000). These receptors comprise several subunits that differentially affect the permeability of each receptor to calcium. In particular, the GluR2 subunit is responsible for making AMPA receptors impermeable to calcium ions, thus ablating toxicity associated with the massive calcium influx that occurs with excitotoxicity (Van Damme et al. 2002). Interestingly, knockdown of this specific subunit in a mouse model of ALS significantly accelerates disease progression while overexpression of GluR2 significantly delays disease onset (Tateno et al. 2004, Van Damme et al. 2005b). Additionally, reduced levels of GluR2 mRNA are observed in spinal cord isolated from ALS patients (Shaw et al. 1999). Collectively, these data suggest that altered expression of AMPA subunits may sensitize motor neurons to excitotoxic injury. There is also substantial evidence that motor neurons in ALS display hyperexcitable activity due to alterations in sodium and potassium ion currents, and thus overreact to excitatory stimuli. For example, depolarization of resting membrane potential by enhanced sodium currents can trigger calcium influx, causing neurons to become more easily excited (Vucic and Kiernan, 2006, van Zundert et al. 2008, Vucic et al. 2009, Vucic and Kiernan, 2010). Alternatively, repolarization of the neuron and restoration of resting membrane potential may be impaired as a result of reductions in outgoing potassium currents such that

neurons remain depolarized following an excitatory stimulus (Bostock et al. 1995, Kanai et al, 2006, Vucic and Kiernan, 2006, Vucic et al. 2009, Wainger et al. 2014). This overreaction to excitatory stimuli is thought to further sensitize motor neurons to excitotoxicity.

Conversely, a number of studies have implicated a loss of inhibitory signaling to motor neurons by interneurons as a potential source of excitotoxicity in ALS. In mice expressing a mutant form of SOD1, for example, inhibitory interneurons are observed to degenerate in parallel with motor neurons (Morrison et al. 1998). Furthermore, inhibitory interneurons in the spinal cord of mice overexpressing TDP-43 or mutant SOD1 are found to accumulate ubiquitinated inclusions in early stages of the disease, resulting in subsequent degeneration, which precedes motor neuron loss (Martin et al. 2007, Wegorzewska et al. 2009). Similar observations have been made in cortical and spinal cord tissue derived from ALS patients, which show reductions in the number of interneurons, although ubiquitination within neurons in these populations is rare (Ince et al. 1993, Stephens et al. 2001, Stephens et al. 2006). These studies correlate with recent data collected from ALS patients demonstrating that intracortical inhibitory signaling is impaired, which could reflect a loss in cortical interneurons (Ziemann et al. 1997, Zanette et al. 2002a, 2002b, Vucic et al. 2009). Taken together, these data may be indicative of a loss of inhibitory signaling to downstream motor neurons, which would normally attenuate the response of these cells to excitatory stimuli. Consequently, in the absence of inhibitory signals in an ALS disease state, motor neurons may overreact to excitatory signals, resulting in excitotoxic injury, and subsequent neuronal death.

1.1.3 The Role of Neuroinflammation in ALS

ALS is commonly thought of as a disease of motor neurons, implying that motor neuron death occurs through pathways that are intrinsic to these cells (i.e. cell-autonomous death); however, in the past two decades, it has become abundantly clear that motor neuron death is driven in large part by deviations in the normal activity of surrounding glial cells within the CNS (i.e. non-cell-autonomous death). Although glial cells, such as astrocytes and microglia, are capable of providing trophic support and protection from foreign pathogens to surrounding neurons, they are also capable of causing significant injury to neighboring cells in response to aversive stimuli. This process, in which glial cells in the CNS become neurotoxic and promote neuronal injury, is known as neuroinflammation.

Neuroinflammation is mediated primarily through the activity of reactive astrocytes and activated microglia. In healthy tissue, astrocytes are involved in providing structural and trophic support to neighboring neurons, in addition to regulating neuronal responses to synaptic transmission (reviewed by Sofroniew and Vinters, 2010). In ALS, however, astrocytes adopt a reactive phenotype in which they secrete a number of toxic factors, including cytokines and chemokines such as interleukin (IL)-6, and withdraw trophic support, ultimately culminating in neuronal death (Farina et al. 2007). Astrocytic activation in ALS has been shown *in vitro* to occur in response to several factors, including GSH depletion within astrocyte cell bodies, and expression of mutant SOD1 (Hensley et al. 2006, Nagai et al. 2007, Marchetto et al. 2008, Lee et al. 2010). Furthermore, there is ample evidence to suggest that astrocytes expressing mutant forms of SOD1 are not only capable of inducing death in co-cultured motor neurons, but also

seem to possess an increased propensity for expression of pro-inflammatory cytokines including TNF-α and Fas ligand (FasL), and display an enhanced expression of these genes in response to cytokine stimulation in comparison to wild type astrocytes (Hensley et al. 2006). Astrocytes derived from neural progenitors isolated from both familial and sporadic ALS patient tissue have also been demonstrated to be neurotoxic to co-cultured motor neurons (Haidet-Phillips et al. 2011). It is also apparent that astrocytes play a significant role in modulating disease progression in ALS. This is most clearly demonstrated by studies of chimeric mice with a selective deletion of G37R mutant SOD1 in astrocytes, which yielded no delay in disease onset and progression, though early disease stage was modestly slowed (Yamanaka et al. 2008). However, late disease progression was significantly delayed. An identical experiment in astrocytes using the G85R form of mutant SOD1, which, unlike G37R SOD1, does not possess any dismutase activity, produced the opposite results, delaying onset and the progression of early disease phase, but not late disease phase (Wang et al. 2011). This apparent conflict can be attributed to differences in the two variants of mutant SOD1 used in these experiments, and indicates that disease progression is not consistent for all familial forms of the disease. Nonetheless, these results indicate that astrocytes participate in ALS disease processes and possess the ability to significantly alter disease progression.

Microglia are the resident immune cells of the brain, possessing a common myeloid lineage with macrophages. These cells exist primarily in a resting state in which they participate in many important functions that are essential to neuronal support and survival, in addition to playing a role in synaptic pruning (Turrin and Rivest, 2006, Paolicelli et al. 2011). When signs of neuronal distress are detected, microglia adopt an

inflammatory phenotype aimed at eliminating foreign invaders or removing damaged cells. While beneficial in the short term, prolonged activation of microglia is capable of causing neuronal injury and death in a manner comparable to that observed with reactive astrocytes. Intriguingly, microglial activation occurs in two stages, which alter throughout ALS disease progression. At disease onset, microglia possess a neuroprotective "M2" phenotype in which cells secrete an abundance of anti-inflammatory factors, such as brain derived neurotrophic factor (BDNF), which aid in promoting neuronal survival (Seeburger and Springer, 1993, Liao et al. 2012). However, as the disease progresses, microglia are known to undergo a deleterious transition to a neurotoxic "M1" phenotype in which the production of pro-inflammatory cytokines and chemokines, such as IL-6, TNF-α, FasL, IL-8, and monocyte chemoattractant protein-1 (MCP-1), is significantly enhanced, and pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), NADPH-oxidase-2 (NOX-2), and cyclooxygenase-2 (COX-2) are upregulated. Notably, many of these factors are found in spinal cord tissue and cerebrospinal fluid from ALS patients, in addition to spinal cord tissue isolated from mice expressing mutant forms of SOD1 (Almer et al. 1999, Almer et al. 2001, Hensley et al. 2003, Henkel et al. 2006, Raoul et al. 2006, Wu et al. 2006, Rentzos et al. 2007, Kuhle et al. 2009, Meissner et al. 2010, Liao et al. 2012). It is also noteworthy that extracellular mutant SOD1 is capable of causing microglial activation and enhancing production of pro-inflammatory cytokines (Zhao et al. 2010). As mutant SOD1 has been shown to be secreted by motor neurons through association with chromogranin A and B, this suggests that microglia in ALS may adopt an inflammatory phenotype in response to mutant SOD1 release in some familial forms of ALS (Urushitani et al. 2006, Zhao et al. 2010).

Like astrocytes, microglia are known to significantly alter disease progression as selective knockout of mutant SOD1 or replacement of mutant SOD1 with wild type SOD1 in cells of myeloid lineage, including microglia, significantly delays progression through the late phase of the disease, though no effect on disease onset is observed (Beers et al. 2006, Boilée et al. 2006). Moreover, increased numbers of microglia have been observed in close proximity to dying motor neurons in spinal cord tissue taken from both sporadic and familial ALS patients (Henkel et al. 2004). Altogether, these data indicate that neuroinflammation mediated by microglia and astrocytes plays a substantial role in modulating the progression of ALS.

1.1.4 The Role of Protein Dysregulation in ALS

Disruptions to proteostasis occur at multiple levels in ALS, evidenced by accumulation and aggregation of mutated proteins, induction of the unfolded protein response (UPR), and perturbances in the clearance of mutant or damaged proteins (Ilieva et al. 2009). As discussed previously, mutant forms of SOD1 and TDP-43, as well as several other mutant proteins known to cause familial forms of ALS such as fused in sarcoma (FUS) and C9orf72, participate in extensive protein aggregation (Mori et al. 2013, Turner et al. 2013). Notably, wild type TDP-43 and FUS aggregation, and aggregation of oxidized wild type SOD1, has also been reported in sporadic cases of ALS (Bosco et al. 2010, Mackenzie et al. 2010). While the mechanisms causing protein aggregation in ALS are not well understood, there is growing evidence that accumulation of these proteins in aggregates plays a major role in inducing cell death by associating with organelles. As previously discussed, accumulation of mutant forms of SOD1 at

mitochondria enhances the propensity of these organelles to produce ROS, contributing to conditions of oxidative stress (Pasinelli et al. 2004). More recently, data have emerged demonstrating that mutant SOD1 also accumulates at the ER, disrupting the ability of this organelle to perform crucial functions in maintaining protein homeostasis (Kikuchi et al. 2006). Ultimately, accumulation of protein aggregates at the ER results in ER stress and induction of the UPR, which, if not corrected, causes cell death.

Expression of markers associated with ER stress are observed in spinal cord motor neurons of transgenic mice expressing mutant forms of SOD1 before protein aggregation, onset of disease symptoms, and muscle denervation (Saxena et al. 2009). Accumulation of SOD1 aggregates at the ER then occurs after disease onset and is observed to increase throughout the progression of the disease, reaching its greatest prevalence at end stage (Kikuchi et al. 2006). Translocation of mutant SOD1 to the ER is associated with induction of the UPR, and mutant SOD1 has been shown to directly interact with binding immunoglobulin protein (BiP), thereby initiating the ER stress response. Caspase-12, which is involved in mediating ER stress-induced apoptosis, has been shown to be activated under these conditions, suggestive of motor neuron death in response to ER stress. Similarly, SOD1 has also been shown to interact with Derlin-1, preventing it from fulfilling its role as a critical mediator of ER- associated degradation of misfolded proteins (Nishitoh et al. 2008). This interaction eventually leads to activation of apoptosis signal-regulating kinase 1 (ASK1) by inhibiting transport of misfolded proteins to the cytoplasm, and death of the cell. This is corroborated by recent findings demonstrating that mutant SOD1 inhibits protein transport from the ER to the Goli apparatus, promoting ER stress and Golgi fragmentation (Atkin et al. 2014). Further

evidence for the involvement of ER stress and the UPR in ALS comes from studies demonstrating that mutant SOD1 mice deficient in pro-death mediators of the UPR such as ASK1, puma, and bim display enhanced motor neuron viability and delays in disease onset and progression (Hetz et al. 2007, Kieran et al. 2007, Nishitoh et al. 2008). As further evidence for the involvement of ER stress in ALS, numerous ER stress markers are shown to be significantly elevated in spinal cord tissue from ALS patients including BiP, protein kinase RNA-like endoplasmic reticulum kinase (PERK), an ER stress sensor, and C/EPB homologous protein (CHOP), a mediator of cell death activated in response to ER stress and the UPR (Ilieva et al. 2007, Atkin et al. 2008, Hetz et al. 2009, Ito et al. 2009, Vajayalakshmi et al. 2011). Furthermore, ER from sporadic ALS patient spinal cords shows pronounced structural abnormalities that may correlate with significant alterations in ER function (Oyangi et al. 2008, Sasaki, 2010).

Aggregation of mutant proteins can also induce cell death though other pathways. Several hypotheses have been put forth to explain the toxicity of such aggregates including proteasome inhibition, sequestration or depletion of chaperone proteins, and general inclusion of vital cellular components (Bruening et al. 1999, Niwa et al. 2002, Urushitani et al. 2002). Intriguingly, inhibition of the proteasome by sustained expression of mutant SOD1 has been demonstrated, as hypothesized by Niwa et al. (2002) with continuous production and degradation of mutant SOD1 'choking' the proteasome and preventing its continued function (Urushitani et al. 2002, Niwa et al. 2002). This could lead to further accumulation of dysfunctional mutant proteins, further aggregation, and ultimately neuronal death. Similarly, overexpression of chaperone proteins such as Hsp70 was shown to offer limited neuroprotection from cell death induced by mutant SOD1,

which might suggest that mutant SOD1 toxicity is in part mediated by a depletion of chaperone proteins (Bruening et al. 1999). Collectively, this evidence suggests that collection of mutant SOD1 into aggregates might function to induce toxicity through a variety of mechanisms unrelated to aggregation phenomena at the mitochondria or in the ER. This is further supported by the observation that reductions in protein aggregation through either genetic or pharmacological upregulation of autophagy has been shown to enhance survival in ALS mice in several recent studies (Hetz et al. 2009, Wang et al. 2012, Castillo et al. 2013).

1.1.5 The Role of Apoptosis in ALS

Though it is evident that ALS is a multi-faceted disease, with many factors contributing to its etiology and progression, these factors ultimately converge at the induction of cell death. In particular, the role of apoptosis, a type of programmed cell death, is well-described in ALS, and many of the processes involved in the disease, such as those discussed above, are known to culminate in activation of pro-apoptotic pathways. Commitment of the cell to apoptosis cannot be reversed; thus modulation of apoptotic signaling pathways represents the final point at which therapeutic intervention can occur to prevent neuronal loss.

Apoptosis can be triggered in a myriad of different ways, but typically proceeds through one of two pathways: the extrinsic apoptotic cascade, which is triggered by binding of pro-apoptotic ligands to death receptors on the cell surface, and the intrinsic apoptotic cascade in which intracellular events trigger the release of apoptogenic factors from mitochondria (Kajta, 2004, Krantic et al. 2005). Extrinsic apoptosis occurs upon

binding of death receptor ligands to their cognate receptors on the plasma membrane, leading to activation of the initiator caspase, caspase-8, which in turn stimulates cleavage and activation of caspase-3, one of three executioner caspases that mediates controlled degradation of vital cellular components, most notably DNA (Sakahira et al. 1998). Pro-inflammatory cytokines, such as TNF-α and FasL, which are produced by enflamed microglia and astrocytes in ALS, are known to bind to death receptors on the plasma membrane, triggering apoptotic cell death in neighboring neurons via the extrinsic apoptotic cascade (Kajta, 2004).

Similarly, events such as oxidative and nitrosative stress, calcium overload caused by excitotoxicity, and ER stress can all induce apoptosis through the intrinsic apoptotic pathway. In this pathway, aberrant calcium signaling, and the accumulation of ROS and RNS cause release of cytochrome c from mitochondria and caspase-9 activation. Together with apoptosis protease-activating factor-1 (APAF-1), these proteins interact to form the apoptosome and activate caspase-3, resulting in death very similar to that observed in the extrinsic cascade (Slee et al. 1999, Luetjens et al. 2000, Ott et al. 2007). ER stress, on the other hand, is believed to activate caspase-8, 9 and 12, causing subsequent activation of caspase 3 and induction of apoptosis (Nakagawa et al. 2000, Jimbo et al. 2003). As caspase 12 levels are known to be elevated in spinal cord from mice expressing mutant SOD1, this suggests that ER stress may play a major role in apoptosis signaling in ALS (Wootz et al. 2004). Apoptosis driven by mitochondria can also proceed in a p53-dependent manner, in which cellular stress causes activation of p53 and its downstream effectors puma, noxa, and Bax, pro-apoptotic Bcl-2 family members (Amaral et al. 2010). Additionally, p53 activity is known to inhibit pro-survival proteins,

such as Bcl-2, levels of which are reduced in spinal cord tissue from ALS patients (Mu et al. 1999). Together, these factors can result in cytochrome c release, caspase activation, and subsequent death. As p53 activity is enhanced in cells overexpressing mutant SOD1 and in brain and spinal cord from ALS patients, activation of this pathway could play a role in promoting neuronal cell death in ALS (Martin, 2000, Barbosa et al. 2010, Ranganathan and Bowser, 2010).

Evidence has also arisen demonstrating that apoptosis in ALS may occur through mitochondrially-linked mechanisms independent of caspase activation. Apoptosis-inducing factor (AIF) is a pro-apoptotic protein released from mitochondria in response to mitochondrial damage and dysfunction (Daugas et al. 2000). Upon release, AIF translocates to the nucleus where is triggers DNA fragmentation and chromatin condensation as a part of apoptotic cell death. Recent studies have found that AIF accumulates in the nuclei of spinal cord motor neurons in mice carrying a mutant SOD1 transgene, and that nuclear AIF levels increase as the disease progresses (Oh et al. 2006). Collectively, these data indicate that AIF likely plays a role in initiating apoptotic cell death of motor neurons in ALS.

The complex nature of ALS has presented a great challenge to the development of effective therapeutics for the disease. In the past, many strategies have targeted only one facet of disease pathology, as is the case with the drug Riluzole; however as our understanding of ALS has grown, so too has the consensus that effective therapeutic strategies must target multiple factors contributing to ALS etiology and progression. In this context, anthocyanins are emerging as promising therapeutic agents for the treatment

of neurodegenerative disease, although their efficacy for the treatment of ALS specifically has not been explored until now.

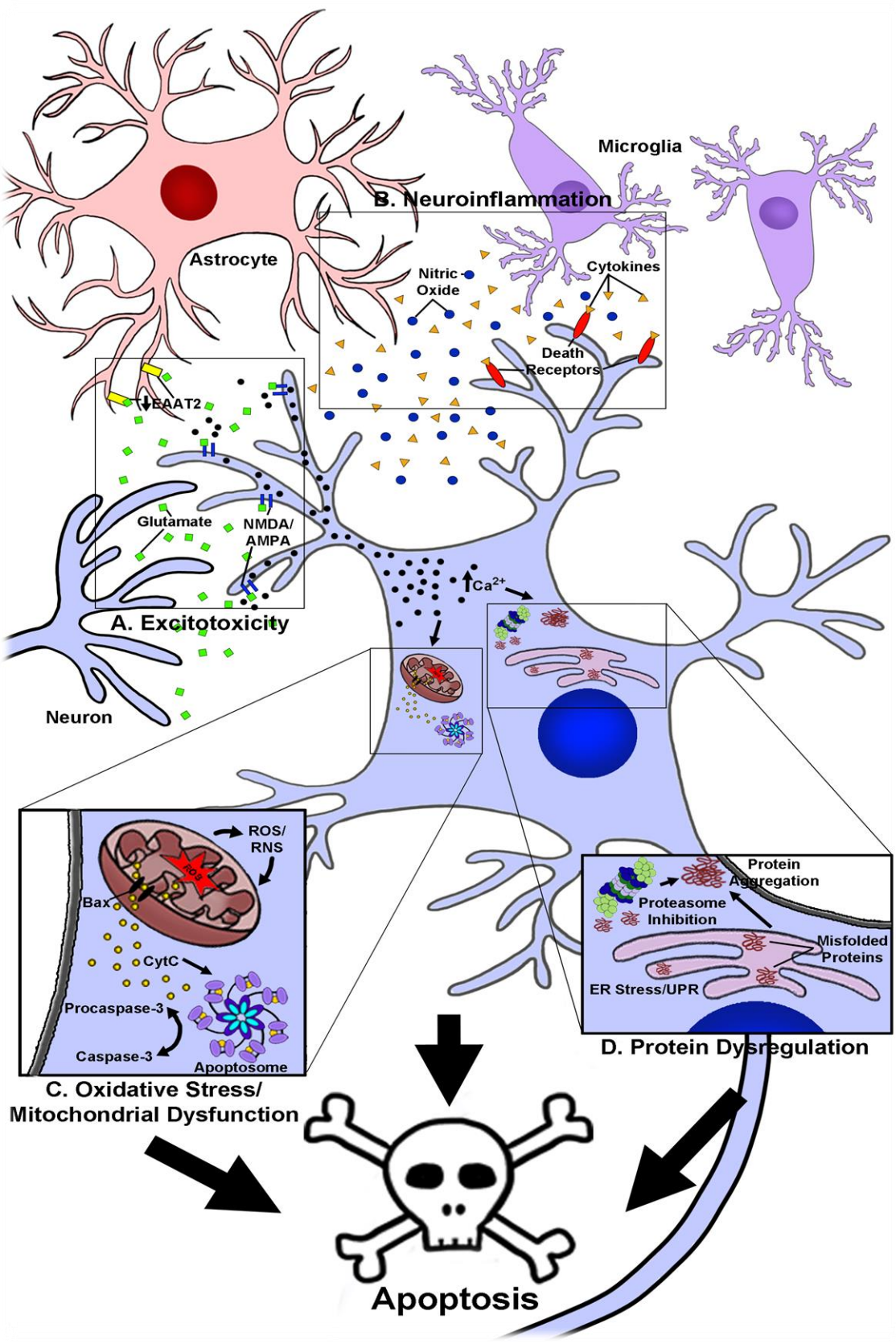
Microglia
B. Neuroinflammation
Astrocyte
Nitric Oxide
Cytokines
Death Receptors
EAAT2
Glutamate
NMDA/ AMPA
A. Excitotoxicity
Ca^{2+}
Neuron
ROS/ RNS
ROS
Bax
CytC
Procaspase-3
Caspase-3
Apoptosome
C. Oxidative Stress/ Mitochondrial Dysfunction
Protein Aggregation
Proteasome Inhibition
Misfolded Proteins
ER Stress/UPR
D. Protein Dysregulation
Apoptosis

Figure 1.1. Molecular mechanisms contributing to ALS pathogenesis. *A,* The role of excitotoxicity in ALS. Glutamate is released from pre-synaptic neuron terminals in elevated quantities and binds to glutamate receptors such as NMDA and AMPA receptors. Receptor binding causes massive calcium influx in post-synaptic neurons and activates pro-apoptotic signaling cascades, in addition to inducing mitochondrial dysfunction and ER stress responses. *B,* The role of neuroinflammation in ALS. Glial cells such as astrocytes and microglia become chronically enflamed in disease states and secrete oxidative species, such as nitric oxide, and pro-inflammatory cytokines. Cytokines bind to death receptors on the cell surface and activate pro-apoptotic signaling cascades. *C,* The role of oxidative stress and mitochondrial dysfunction in ALS. Mitochondrial dysfunction occurs as a result of several factors in ALS, causing mitochondria to produce elevated levels or reactive oxygen and nitrogen species (ROS and RNS). Enhanced production of ROS and RNS exacerbates mitochondrial dysfunction, eventually causing release of the pro-apoptotic signaling protein, cytochrome c (CytC). Cytochrome C contributes to formation of the apoptosome, which in turn cleaves procaspase-3 to form active caspase-3, stimulating apoptosis. *D,* The role of protein dysregulation in ALS. ER stress occurs as a result of multiple factors in ALS, causing accumulation of misfolded proteins and activation of the unfolded protein response (UPR). As misfolded and aggregated proteins accumulate, the proteasome becomes clogged, leading to proteasome inhibition and further accumulation of protein aggregates. Protein aggregation and ER stress trigger pro-apoptotic signaling cascades. Collectively, these factors lead to death of motor neurons.

1.2 The Use of Anthocyanins as Novel Neuroprotective and Therapeutic Agents in Neurodegenerative Disease

Flavonoids are a large class of polyphenolic compounds found in many fruits, vegetables, spices and herbs that are best known for their high levels of intrinsic antioxidant activity. However, in recent years the beneficial effects of flavonoid-rich diets have been found to influence a diverse array of functions, including modulation of inflammatory and apoptotic signaling pathways. Anthocyanins in particular have garnered significant attention in this regard, and reports describing their therapeutic benefit for a variety of conditions such as cardiovascular disease and cancer have emerged (reviewed by Wallace 2011, Wang and Stoner 2008). These unique cationic compounds are found in high concentrations in a number of fruits, vegetables, and flowers, and are responsible for creating the red, blue, and purple pigmentation observed in many of these species. Common anthocyanins are composed of one of six anthocyanidin bases, which differ in molecular structure at the B-ring, and a sugar moiety attached at the third position of the C-ring (Fig. 1.2). They are appealing as therapeutic agents for many reasons, one of the most notable being that they are among the most commonly consumed flavonoids in a normal diet and, for this reason, they are generally recognized as safe (Scalbert and Williamson, 2000).

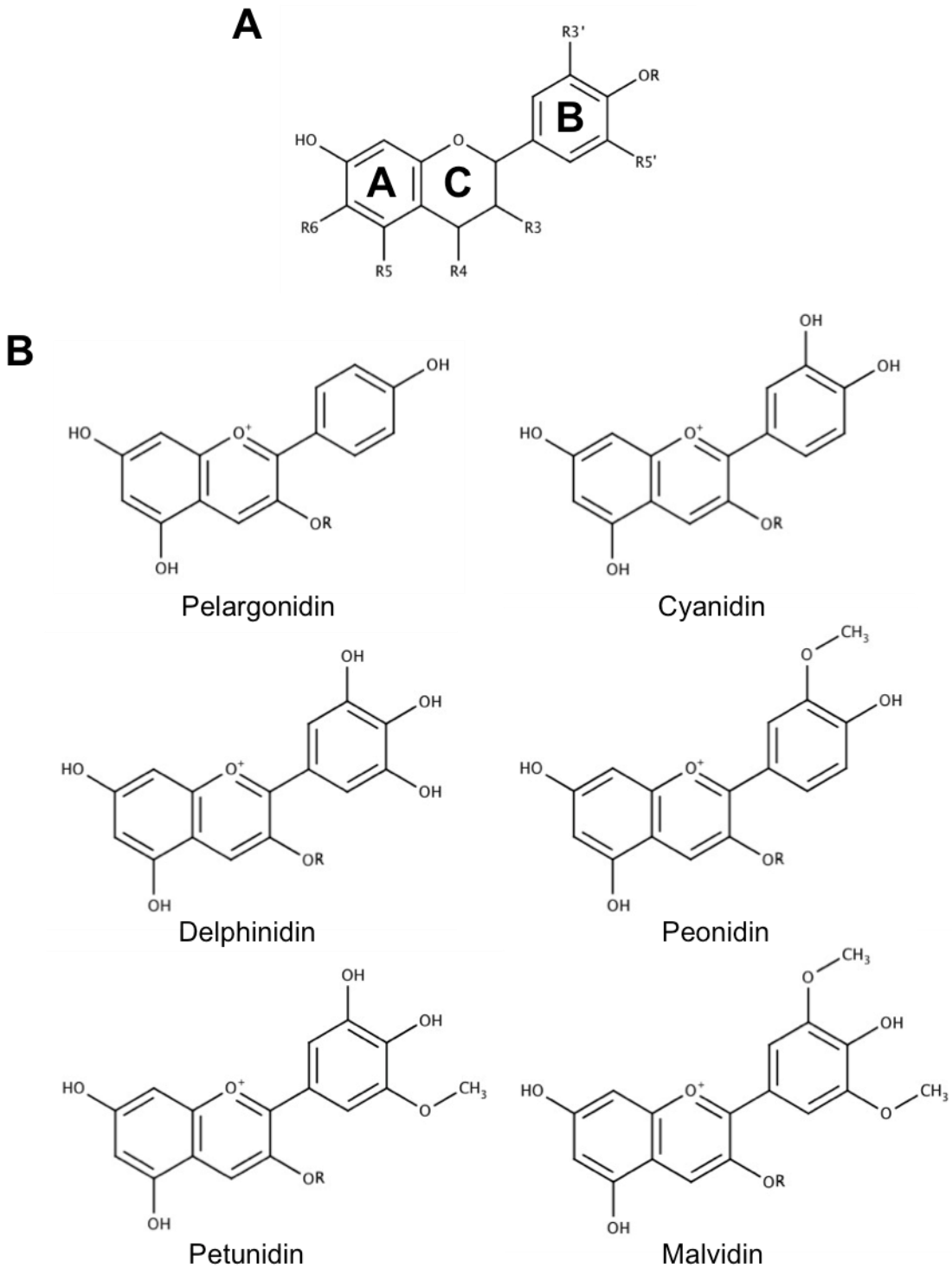

Figure 1.2. Common anthocyanin structures. *A,* General flavonoid structure. Flavonoids possess a characteristic three-ring structure that is conserved across all family members. Several classes of flavonoids exist, including anthocyanins, which differ depending on substitutions of the A, B, and C-rings. *B,* Structures of the six most

common anthocyanins. Anthocyanins possess a cationic structure that differs between species predominately in substitutions of the B-ring. Anthocyanins also possess a sugar moiety as a part of their structure, represented as R. Common sugar moieties include but are not limited to glucose, galactose, and rutinose. All structures included in this dissertation were created using MarvinSketch (ChemAxon, Cambridge, MA).

The ability of anthocyanins to attenuate disorders of the CNS is currently under exploration, although data regarding their effects on neurodegeneration are still relatively limited. Nevertheless, several findings have emerged to suggest that anthocyanins ameliorate many of the damaging effects of processes implicated in neurodegeneration such as oxidative and nitrosative stress, excitotoxicity, glial inflammation, protein aggregation, and induction of apoptotic signaling proteins. Furthermore, evidence has accumulated showing that anthocyanins are capable of crossing the blood brain barrier (BBB), suggesting that these compounds may mediate these effects directly in the CNS where neuronal death takes place (Youdim et al. 2003, Andres-Lacueva et al. 2005, El Mohsen et al. 2006, Williams et al. 2008). Limited data have also been reported demonstrating that anthocyanins may be effective therapeutic agents for Parkinson's disease, Alzheimer's disease, and aging, which share many common pathological features with ALS. These studies are discussed in detail below.

1.2.1 Absorption and Blood Brain Barrier Permeability of Anthocyanins

Following ingestion, anthocyanins are swiftly taken up and absorbed into the blood stream where they are transported to target tissues (El Mohsen et al. 2006, Milbury and Kalt, 2010, Vanzo et al. 2011). The rapid kinetics of anthocyanin absorption into systemic circulation suggests that initial absorption likely takes place in the stomach (Passamonti et al. 2003). This process is thought to be mediated by a bilitranslocase transporter as anthocyanins have been shown to interact with this transporter in several studies (Maestro et al. 2010, Vanzo et al. 2008). A similar mechanism is thought to be responsible for the uptake of these compounds into the CNS as bilitranslocase, possessing

a bilirubin binding motif, is present in the endothelial cells that help form the BBB (Battiston et al. 1999). Furthermore, the interaction between bilitranslocase and anthocyanins is mediated by hydrogen bonding, which provides a compelling level of specificity for this mechanism. There is also some evidence that flavonoids, such as anthocyanins, may interact with p-glycoprotein transporters, and gain entrance into the brain in this manner (Youdim et al. 2004).

Upon transport into the CNS, anthocyanins have been shown to accumulate in brain tissue at levels up to 0.21nmol/g of tissue in rodent models (Passamonti et al. 2005; Talavera et al. 2005). Accumulation occurs in several tissues, including brain endothelial cells, brain parenchymal tissue, as well as striatum, hippocampus, cerebellum, and cortex (Youdim et al. 2003, Andres-Lacueva et al. 2005, El Mohsen et al, 2006). This finding is of particular interest in that several of these brain regions are known to contain vulnerable neuronal populations whose loss is implicated in several forms of neurodegenerative disease (Andres-Lacueva et al. 2005).

1.2.2 Antioxidant Effects of Anthocyanins

Anthocyanins, like many flavonoids, are unique antioxidants in that they are able to scavenge damaging ROS and RNS directly, as evidenced by their high oxygen radical absorption capacity (ORAC) values, in addition to enhancing the cell's intrinsic antioxidant defenses (Shih et al. 2007, Zafra-Stone et al. 2007, Zhu et al. 2010, Hwang et al. 2011). Direct scavenging of various ROS including 1,1-diphenyl-2-picrylhydrazyl (DPPH), alkyl, and hydroxyl radicals has been reported using electron spin resonance spectroscopy (Hwang et al. 2012). Similarly, the major anthocyanin constituents of plum

extract have been shown to scavenge superoxide radicals at a higher capacity than other flavonoids such as quercetin, which is thought to play a role in mediating the neuroprotective effects of these compounds (Chun et al. 2003). Indeed, many studies utilizing both anthocyanin-rich fruit extracts and pure anthocyanins *in vitro* have demonstrated that these compounds provide potent protection from hydrogen peroxide toxicity in a variety of neuronal cell lines (Heo and Lee 2005, Ghosh et al. 2006, Tarozzi et al. 2007, Spada et al. 2009). As both superoxide and hydrogen peroxide are the products of many physiological processes, and are known to be elevated in the context of neurodegeneration by processes such as mitochondrial dysfunction and glial inflammation, the ability of anthocyanins to scavenge these ROS suggests that they may be well suited to ameliorate oxidative damage in diseases like ALS. This is further supported by an *in vivo* study in which rats were injected with carbon tetrachloride, an agent that causes generation of free radicals and oxidative damage within several tissues, including brain, after chronic consumption of anthocyanin-rich grape juice (Dani et al. 2008). Rats that received grape juice supplementation displayed a significant reduction in markers of oxidative damage such as lipid peroxidation and protein carbonylation compared to rats treated with carbon tetrachloride alone.

Indirect mitigation of oxidative and nitrosative stress by anthocyanins occurs in several ways and is due primarily to an increase in the levels and activities of antioxidant enzymes. Several reports have documented that treatment with pure anthocyanins or anthocyanin-rich extracts enhances levels of the enzymes catalase, which scavenges hydrogen peroxide, and SOD1 both *in vitro* and *in vivo* (Heo and Lee 2005, Ghosh et al. 2006, Dani et al. 2008, Tarozzi et al. 2007, Spada et al. 2009, Poulose et al. 2016).

Additionally, anthocyanins have been shown to enhance levels of GSH within neuronal cultures in addition to directly enhancing the activity of glutathione peroxidase, which plays a vital role in detoxifying hydrogen peroxide using GSH (Kelsey et al. 2011). Moreover, both pure anthocyanins and anthocyanin-rich extracts have been shown to reduce mitochondrial oxidative stress and dysfunction induced by either Bcl-2 inhibition or rotenone toxicity (Kelsey et al. 2011, Strathearn et al. 2014). These activities are thought to be modulated in part by the ability of anthocyanins to induce nuclear factor erythroid 2-related factor 2 (Nrf2) activity, which acts as a master regulator of many antioxidant genes including catalase and the regulatory subunit of gamma-glutamylcysteine ligase, the enzyme responsible for carrying out the rate limiting step of GSH synthesis, in addition to a host of other phase II detoxification enzymes (Hwang et al. 2011, Shih et al. 2007). This is supported by a recent study demonstrating that aged rats fed an anthocyanin-rich acai pulp diet showed significantly elevated Nrf2 levels in hippocampus and prefrontal cortex in addition to enhanced levels of antioxidant enzymes such as SOD1 and glutathione S-transferase (Poulose et al. 2016). Collectively, these data suggest that the neuroprotective effects of anthocyanins are mediated through both direct and indirect antioxidant activities within the brain (Fig 1.3).

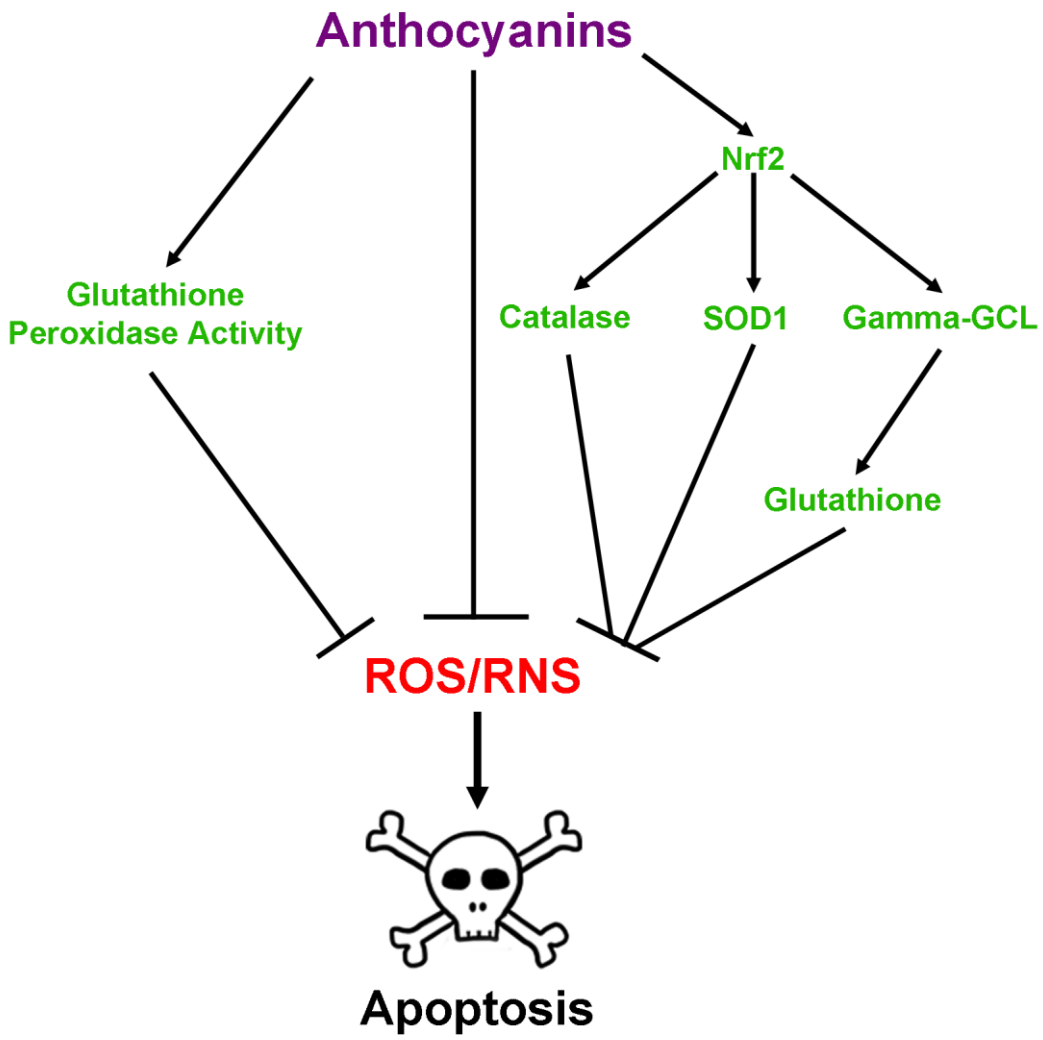

Figure 1.3. Antioxidant effects of anthoycanins. Anthocyanins modulate damage produced by reactive oxygen and nitrogen species (ROS and RNS) by several mechanisms. These include direct enhancement of glutathione peroxidase activity, direct scavenging of ROS and RNS, and activation of Nrf2 transcription of antioxidant enzymes. Genes activated by Nrf2 include those for catalase, Cu, Zn-superoxide dismutase (SOD1), and gamma-glutamylcysteine ligase (γ-GCL), which increases synthesis of the critical antioxidant, glutathione. Collectively, these mechanisms detoxify ROS and RNS to prevent apoptosis.

1.2.3 Anthocyanins in Calcium Homeostasis and Excitotoxicity

Maintaining calcium homeostasis is an essential part of preventing neuronal cell death induced by excitotoxicity. Although the mechanism by which anthocyanins preserve calcium homeostasis is not well understood, several lines of evidence have recently emerged demonstrating that these compounds have significant effects on calcium flux in response to many neurotoxic agents. For example, cells treated with amyloid beta, one of the neurotoxic species thought to underlie the development of Alzheimer's disease, display significant disturbances in calcium homeostasis leading ultimately to excitotoxicity. However, several studies have reported that anthocyanin treatment in both neuronal cell lines and primary hippocampal cells prevents increases in intracellular calcium caused by this insult (Ye et al. 2010, Shih et al. 2011, Badshah et al. 2015). Anthocyanins have also been shown to promote restoration of calcium levels following depolarization induced by dopamine treatment in primary rat hippocampal neurons (Poulose et al. 2014).

The direct effects of anthocyanins on excitotoxic insult have also been described both *in vitro* and *in vivo*. In one study, anthocyanins were shown to attenuate increases in intracellular free calcium in both primary hippocampal cells and the HT22 hippocampal cell line in response to treatment with kainate, a glutamate receptor agonist (Ullah et al. 2014). This effect was responsible in part for reducing excitotoxic cell death in these cells. In a similar manner, treatment with the pure anthocyanin, cyanidin-*O*-3-glucoside, inhibited glutamate-induced increases in calcium concentration in rat hippocampal neurons (Yang et al. 2015). These effects have also been observed *in vivo* using a model of retinal ganglion cell degeneration. Intraocular injection of mice with N-methyl-D-

aspartate (NMDA) induced excitotoxicity by stimulating calcium influx through ionotropic glutamate receptors, causing wide-spread cell death in injected mice; however simultaneous injection with anthocyanin-rich bilberry extract dramatically attenuated these effects, demonstrating that anthocyanins may be capable of mitigating excitotoxicity *in vivo* (Fig 1.4; Matsunaga et al. 2009).

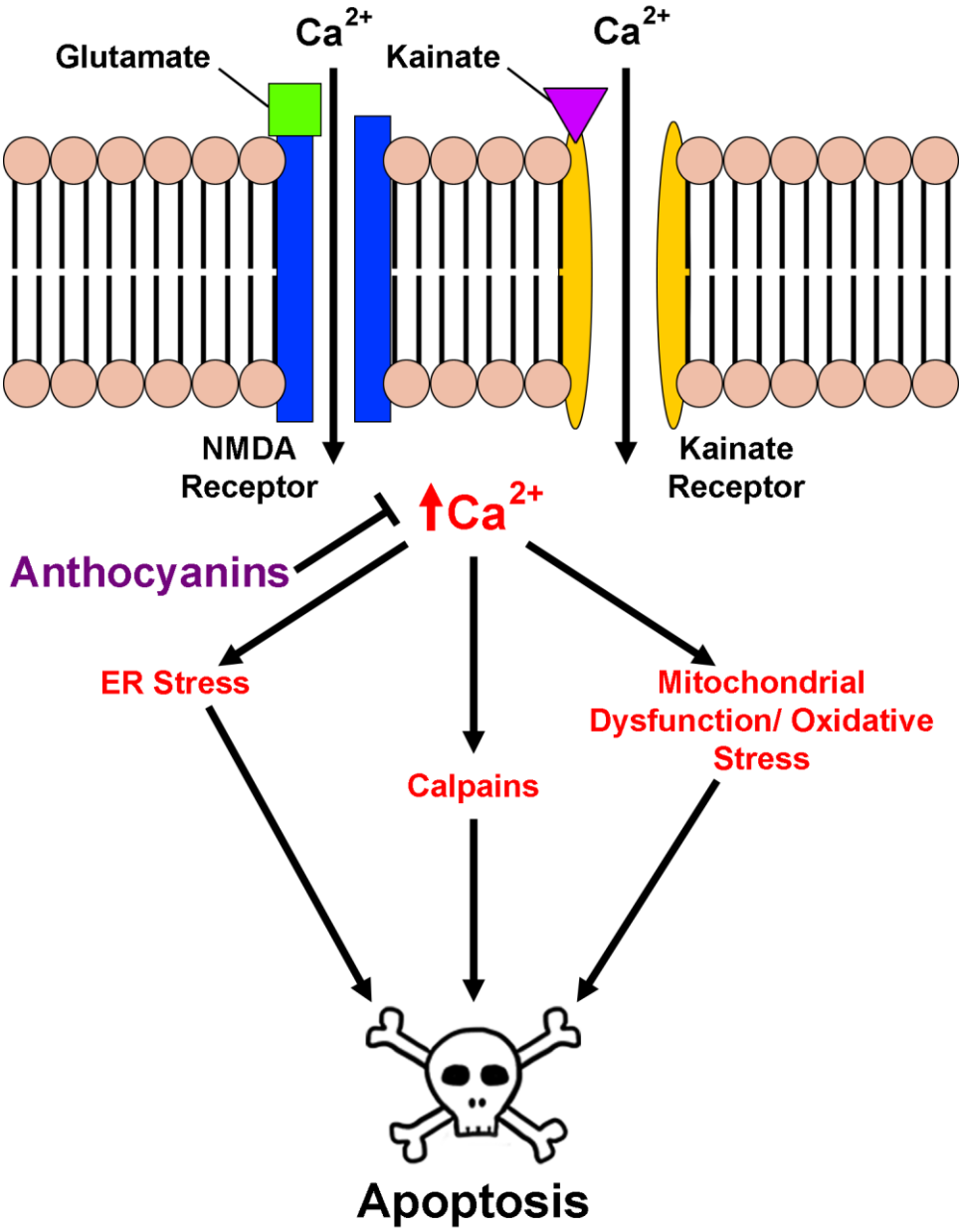

Figure 1.4. Effects of anthocyanins on calcium homeostasis and excitotoxicity. Binding of the excitatory compounds, glutamate or kainate, to their cognate receptors on the cell membrane causes massive calcium influx into neurons, causing ER stress, mitochondrial dysfunction and oxidative stress, and activation of pro-apoptotic factors such as calpain, leading to cell death. Anthocyanins protect neurons from excitotoxicity by preventing increases in intracellular calcium caused by glutamate and kainate signaling.

1.2.4 Anti-neuroinflammatory Activity of Anthocyanins

The anti-inflammatory effects of anthocyanins are well established in non-neuronal systems, particularly those related to cardiovascular disease (reviewed by Wallace, 2011). However, their role in reducing neuroinflammation is less understood and the topic of current exploration. Several recent studies have assessed the anti-inflammatory effects of anthocyanins and anthocyanin-rich extracts on the BV2 mouse microglial cell line. These studies have demonstrated that anthocyanin treatment is capable of significantly reducing induction of pro-inflammatory proteins such as iNOS and COX-2 in response to stimulation with lipopolysaccharide (LPS), a component of bacterial cell walls that is known to induce a pronounced inflammatory response. Furthermore, these studies demonstrated that treatment with anthocyanin-rich extracts significantly attenuates production and secretion of nitric oxide, IL-1β and TNF-α (Lau et al. 2007, Poulose et al. 2012, Carey et al. 2013, Jeong et al. 2013). Modulation of pro-inflammatory signaling pathways is also reported as levels of active c-Jun-N-terminal kinase (JNK), p38-mitogen activated protein kinase (p38-MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), and Akt, are all significantly reduced (Lau et al. 2007, Poulose et al. 2012, Carey et al. 2013, Jeong et al. 2013). Reduction in the activity of these signaling pathways correlates with reductions in activated nuclear factor-κB (NF-κB), and prevention of its translocation to the nucleus, where it is capable of mediating the transcription of many pro-inflammatory genes. Though few in number, these studies clearly demonstrate that treatment with anthocyanins or anthocyanin-rich extracts is capable of ameliorating many of the inflammatory effects of microglial activation.

These findings are mirrored *in vivo* in an experiment demonstrating that mice receiving anthocyanins from purple sweet potatoes displayed a marked decrease in neuroinflammatory markers following injection with LPS (Wang et al. 2010). Levels of both iNOS and COX-2 were significantly reduced in the brains of anthocyanin-treated mice, correlating with significant improvements in cognition and memory tasks. Other studies have indicated that anthocyanin-rich extracts from acai and black soybeans effectively attenuate inflammation induced in models of natural and artificial aging, the latter achieved by treatment with D-galactose (Poulose et al. 2016, Rehman et al. 2016). Acai-rich diets significantly reduced levels of both NOX-2 and NF-κB in a model of natural (chronological) aging, in good agreement with *in vitro* studies with isolated microglia (Poulose et al. 2016). Black soybean anthocyanin extract displayed similar results in a D-galactose model of neuroinflammation and artificial aging as rats receiving anthocyanin treatment displayed reduced levels of iNOS and NF-κB activity, as well as reductions in TNF-α and ROS production, and lipid peroxidation. Moreover this study reported reduced levels of reactive astrocytes and activated microglia in hippocampal tissue of anthocyanin-treated rats, indicating that these unique compounds are capable of exerting anti-inflammatory effects on glial cells *in vivo* (Fig 1.5; Rehman et al. 2016).

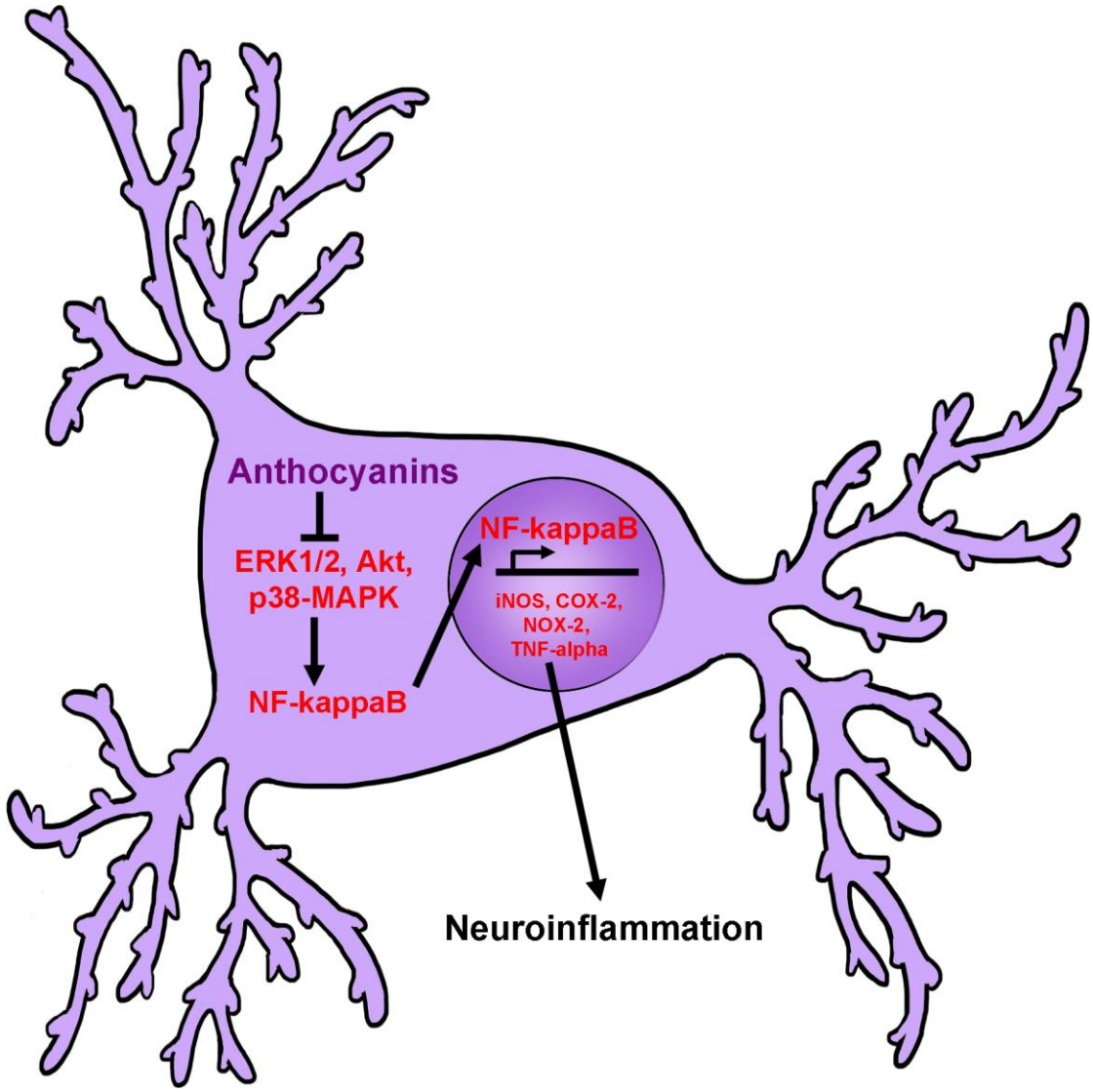

Figure 1.5. Effects of anthocyanins on neuroinflammation. Inflammatory stimuli cause activation of extracellular regulated signal kinase 1/2 (ERK1/2), Akt, and p38-mitogen-activated protein kinase (p38-MAPK), which subsequently activate nuclear factor-κB (NF-κB) in microglia. NF-κB then translocates to the nucleus and initiates transcription of pro-inflammatory genes including, but not limited to inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) NADPH-oxidase-2 (NOX-2), and tumor necrosis factor-α (TNF-α). It is thought anthocyanins inhibit this pathway by blocking activation of ERK1/2, Akt, and p38-MAPK.

1.2.5 Anthocyanins and Regulation of Protein Homeostasis

Though limited, new data are emerging to suggest that anthocyanins may also exert neuroprotective effects by directly preventing protein aggregation and by stimulating autophagy. As protein aggregation has been suggested to play a significant role in neuronal death for many diseases, the ability of therapeutic agents to inhibit protein oligomerization into toxic plaques and fibrils is desirable (Table 1.1). The pure anthocyanin cyanidin-*O*-3-glucopyranoside has been shown to directly interfere with oligomerization of amyloid beta peptides, one of the major constituents of senile plaques observed predominately in Alzheimer's disease (Tarozzi et al. 2010). Similarly, the aglycon, malvidin, and its glucoside conjugate have also been reported to potently inhibit amyloid beta oligomerization into toxic fibrils (Rivière et al. 2008). Though the mechanism by which anthocyanins inhibit aggregate formation is currently unknown, the ability of these compounds to disrupt the formation of toxic oligomers is promising for their therapeutic efficacy. Further data is needed to determine if anthocyanins are also able to disrupt toxic aggregate formation of other protein species such as mutant SOD1.

Table 1.1. Protein aggregation in neurodegenerative disease.

Neurodegenerative Disease	Major Constituents of Protein Aggregates	Reference
Alzheimer's Disease	Amyloid Beta Peptide (from APP)	Glenner and Wong, 1984 Masters et al. 1985 Selkoe et al. 1986
	Hyperphosphorylated Tau	Bancher et al. 1989
Parkinson's Disease	α-synuclein	Baba et al. 1998
ALS	C9orf72	Mori et al. 2013
	FUS	Vance et al. 2009 Ling et al. 2010
	SOD1	Burijn et al. 1998 Bosco et al. 2010
	TDP-43	Johnson et al. 2009 Mackenzie et al. 2007

Recent studies have also reported the ability of anthocyanin-rich extracts from acai fruit pulp to modulate autophagy. This process, in addition to proteasomal degradation, is vital for clearing toxic aggregates and misfolded proteins from the intracellular space (Poulose 2014, Poulose et al. 2016). Treatment of HT22 cells with the autophagy inhibitors wortmanin and bafilomycin A1 caused significant accumulation of poly-ubiquitinated proteins, which was corrected by treatment with acai pulp extracts (Poulose 2014). Additionally, these extracts significantly enhanced turnover of autophagosomes and enhanced activation of mammalian target of rapamycin (mTOR), one of several regulators of the autophagy pathway. These results were confirmed *in vivo* in the brains of aged rats treated with acai pulp extracts, demonstrating upregulation of autophagy markers, such as mTOR activation (Poulose et al. 2016). Taken together, these results suggest that anthocyanins and anthocyanin-rich extracts may modulate processes such as protein aggregation and autophagy to correct disruptions in protein homeostasis observed in neurodegenerative disease, though further data is needed to confirm this hypothesis.

There is also some evidence to suggest that anthocyanins may regulate ER stress *in vivo*, thereby preventing ER stress-induced apoptosis. In a mouse model of cognitive impairment induced by domoic acid, mice experienced a significant increase in levels of ER stress markers such as PERK and ASK1 in hippocampal tissue (Lu et al. 2012). However, treatment with purple sweet potato extract entirely mitigated this effect, returning these markers to normal levels and preventing ER stress-induced apoptosis. Though this is the only study to date exploring the effect of an anthocyanin-rich extract on neuronal ER stress *in vivo*, it demonstrates that these compounds may show some

benefit in disorders like ALS for which ER stress and concomitant protein dysregulation is an underlying factor (Fig. 1.6).

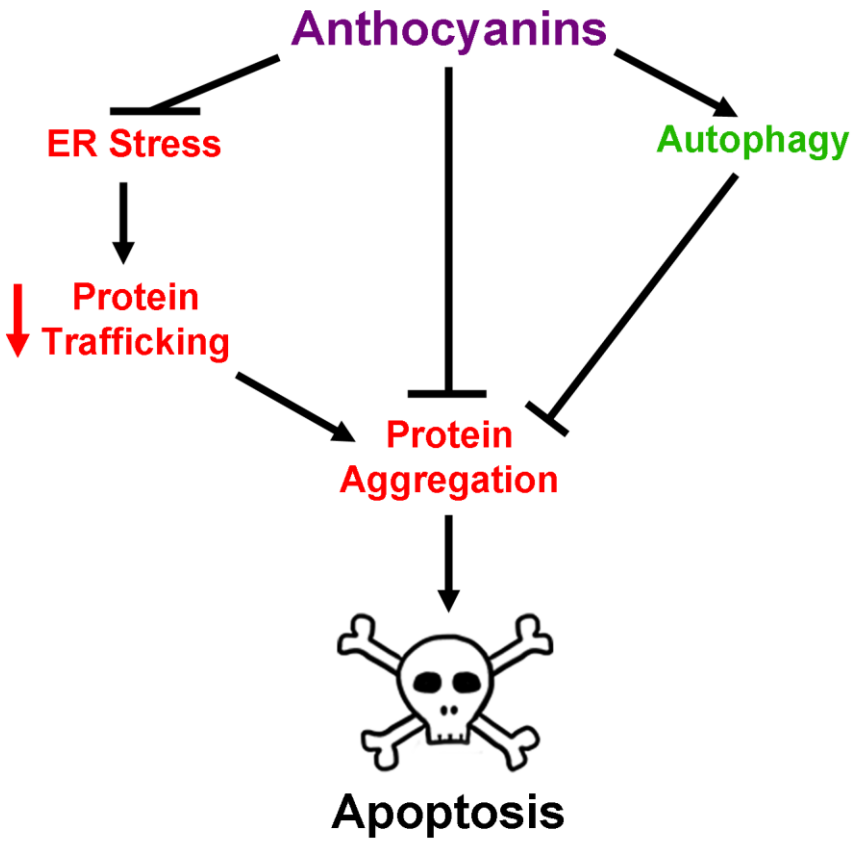

Figure 1.6. Effects of anthocyanins on protein dysregulation and homeostasis. Protein homeostasis is disrupted in several ways in neurodegenerative disease. Increased levels of ER stress cause significant decreases in protein trafficking to other organelles such as the Golgi apparatus. Decreased protein trafficking results in accumulation of misfolded and mutant proteins, causing protein aggregates to form. Formation of these toxic aggregates then contributes to induction of neuronal apoptosis. Anthocyanins modulate this process by reducing ER stress, directly inhibiting the formation of toxic protein aggregates, and stimulating autophagy processes to clear aggregates formed within the neuron.

1.2.6 Anti-apoptotic Effects of Anthocyanins

Anthocyanins are known to modulate several signaling pathways involved in cell death and survival. Their effects on apoptosis specifically are well documented and have been shown to occur in both caspase-dependent and caspase-independent manners (Reddivari et al. 2007). For example, in a D-galactose mouse model of aging, treatment with purple sweet potato extract suppressed activation of pro-apoptotic proteins such as JNK while also preventing mitochondrial release of cytochrome c and subsequent execution of apoptotic signaling pathways (Lu et al. 2010, Ye et al. 2010). These effects were mediated by activation of phosphoinositide-3-kinase (PI3K), which is an upstream activator of Akt, a major regulator of pro-survival signaling. In agreement with these findings, it was also reported that anthocyanins attenuate p53 and JNK-dependent apoptosis in a model of ischemic injury induced by cerebral artery occlusion, which shares many pathological features with neurodegenerative disease such as oxidative stress, excitotoxicity, neuroinflammation, and ultimately neuronal death (Shin et al. 2006). There are also reports that anthocyanins are capable of modulating the expression of Bcl-2 family members. Indeed, in a cellular model of 6-hydroxydopamine (6-OHDA) toxicity, a chemical often used to induce a Parkinsonian phenotype in mice and rats, treatment with an anthocyanin-rich extract from mulberry elevated expression of pro-survival Bcl-2 protein while suppressing expression of the pro-apoptotic Bax protein (Kim et al. 2010). Finally, anthocyanins have also demonstrated the ability to prevent release of AIF from mitochondria, likely accounting for the ability of these compounds to effectively mitigate caspase-independent apoptosis (Min et al. 2011). In sum, these data indicate that anthocyanins are not only capable of ameliorating many factors implicated

in causing neuronal death in ALS, but also pro-apoptotic signaling itself, thus targeting disease progression at multiple levels (Fig. 1.7).

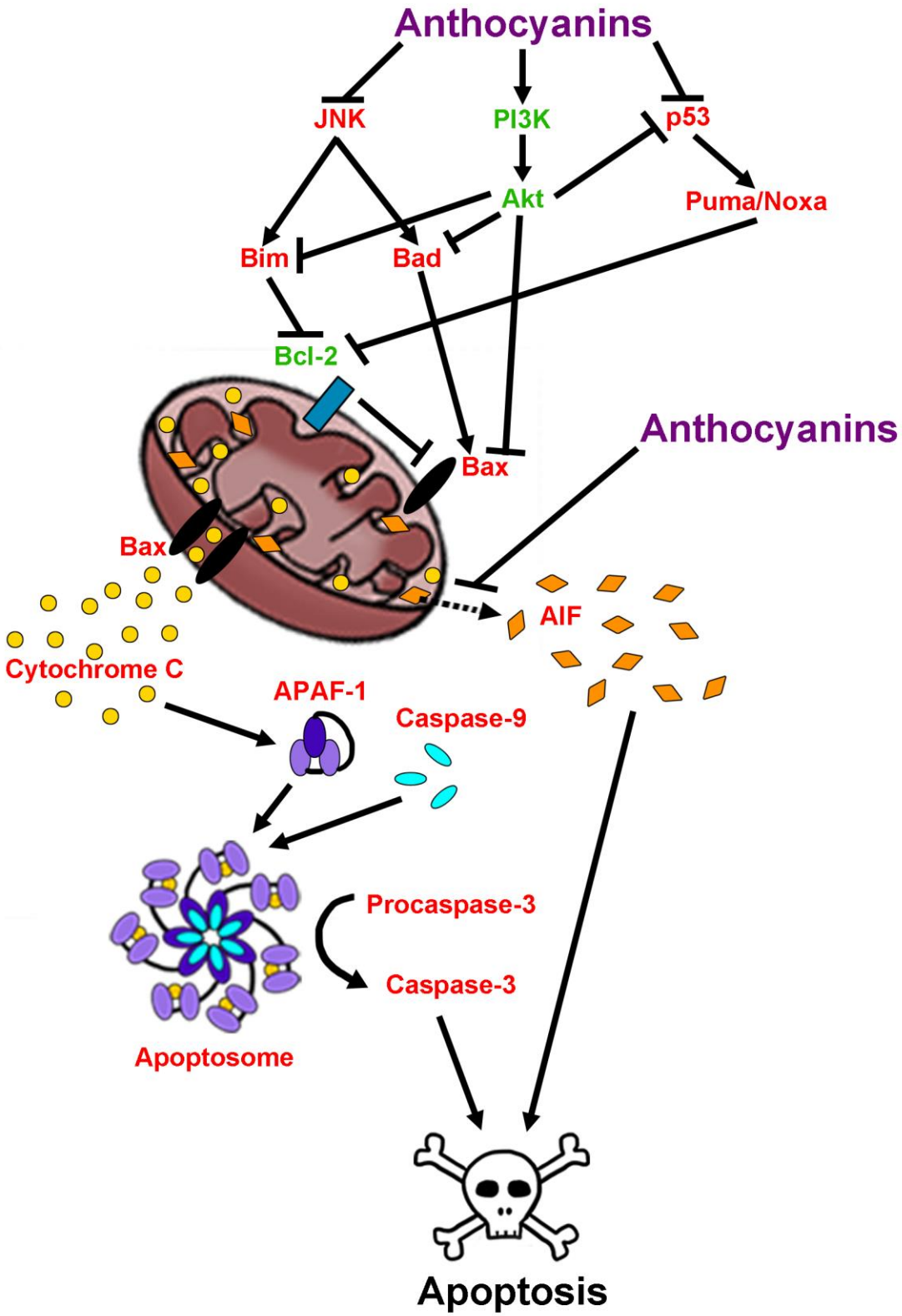
Anthocyanins
JNK
PI3K
p53
Akt
Puma/Noxa
Bim
Bad
Bcl-2
Anthocyanins
Bax
Bax
AIF
Cytochrome C
APAF-1
Caspase-9
Procaspase-3
Caspase-3
Apoptosome
Apoptosis

Figure 1.7. Effects of anthocyanins on pro-survival and pro-apoptotic signaling pathways. Anthocyanins modulate several signaling pathways involved in cell survival and death. Anthocyanins inhibit the activity of c-Jun N-terminal kinase (JNK) and p53, which are responsible for activating pro-apoptotic family members of the Bcl-2 family of proteins, Bim, Bad, Puma, and Noxa. Bim, Puma, and Noxa are known to inhibit the pro-survival functions of Bcl-2, causing activation of the pro-apoptotic protein, Bax. Bax can also be activated by interaction with Bad. Bax then forms pores in the mitochondrial membrane, allowing the release of cytochrome c from mitochondria. Cytochrome c interacts with apoptosis protease activating factor-1 (APAF-1) and caspase-9 to form the apoptosome. The apoptosome then cleaves procaspase-3 to form active caspase-3, stimulating apoptosis. Anthocyanins also enhance the activity of the phosphoinositide-3-kinase (PI3K)/Akt pro-survival signaling pathway, which inhibits activity of pro-apoptotic Bcl-2 family members including Bim, Bad, and Bax, in addition to inhibiting the activity of p53. This activity inhibits entry of neurons into caspase-dependent apoptosis. Alternatively, anthocyanins have also been shown to inhibit caspase-independent apoptosis by blocking the translocation of apoptosis inducing factor (AIF) from mitochondria to the cytosol and subsequently, the nucleus (dashed arrow).

1.2.7 Anthocyanins as Therapeutic Agents in Neurodegenerative Disease Models

Since anthocyanins display impressive pleiotropic effects, combating multiple facets of neurodegeneration, it has been recently hypothesized that they may be effective therapeutic agents for the treatment of neurodegenerative disease. Though no studies have been conducted in models of ALS specifically, several reports have indicated that anthocyanin-enriched extracts are capable of relieving the motor and cognitive deficits associated with Parkinson's disease and Alzheimer's disease, respectively.

Parkinson's disease is often modeled chemically using injection of the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which has been shown to induce the death of nigrostriatal dopaminergic neurons, the major neuronal population affected in Parkinson's disease. In mice that were treated with mulberry extract before MPTP injection, however, this effect was significantly reduced and dopaminergic neurons were preserved (Kim et al. 2010). This observation correlated with decreases in pro-apoptotic protein expression and significant improvements in Parkinsonian symptoms. A similar effect was described in another chemical model of Parkinson's disease using rats injected with 6-OHDA. Studies using 6-OHDA have demonstrated that this compound causes significant oxidative damage and neuronal death in relevant neuronal populations associated with Parkinson's disease (Simola et a. 2007). Rats injected unilaterally with 6-OHDA showed decreased numbers of dopaminergic neurons within the lesioned *substantia nigra* and elevation in levels of lipid peroxidation (Roghani et al. 2010). These effects were essentially ablated, however, by administration of the aglycon anthocyanin derivative, pelargonidin, which corresponded to significant improvements in motor function.

Positive findings have also been shown for the treatment of Alzheimer's disease and age-related cognitive impairment with anthocyanin-rich extracts. A recent study in the APP/PS1 mutant mouse model of Alzheimer's disease, which expresses the transgenes for both mutant amyloid precursor protein (APP) and mutant presenilin-1 (PS1), demonstrated that transgenic mice develop significant cognitive impairments in spatial working memory and accumulation of amyloid beta in brain tissue (Vepsäläinen et al. 2013). This accumulation was significantly reduced by treatment with anthocyanins isolated from bilberry and black currant, and both extracts were able to prevent cognitive decline and improve behavioral abnormalities. Similarly, a recent study by Qin et al. (2013) demonstrated that treatment with cyanidin-O-3-glucoside prevented cognitive impairment induced by amyloid beta intra-cerebro-ventricular injection. Positive findings were also reported by Badshah et al. (2015) in anthocyanin-treated rats injected with amyloid beta, which showed significant reductions in markers of apoptosis and Alzheimer's disease. These findings are in good agreement with a study conducted in senescence accelerated mouse prone 8 (SAMP8) mice, which are used as a model of accelerated aging. These mice experience significant cognitive impairment, which correlates with deposition of amyloid beta plaques, reminiscent of those observed in Alzheimer's disease. Additionally, these mice display elevated levels of stress kinase signaling in the brain mediated by JNK and p38-MAPK. Treatment with an anthocyanin-rich extract from mulberry preserved cognitive function and significantly reduced amyloid plaque burden and stress kinase signaling in the brains of treated mice (Shih et al. 2010). Human studies in older adults at risk for dementia have also yielded promising results with regards to anthocyanin consumption. The dietary introduction of blueberries

and Concord grape juice, which are rich in anthocyanins, significantly improved mild memory impairment in treated individuals (Krikorian et al 2010a, 2010b).

While preclinical and clinical findings on the use of anthocyanins for the treatment of neurodegenerative disease are currently limited in number, they provide compelling evidence for the potential therapeutic efficacy of these compounds. Moreover, as diseases such as Parkinson's disease and Alzheimer's disease share many common pathogenic features with ALS, these studies strongly suggest that anthocyanins may also show beneficial effects in preclinical models of this disease. As such, further study into the mechanism of action and effectiveness of these compounds is warranted.

1.3 The Use of Anthocyanin Metabolites as Novel Neuroprotective and Therapeutic Agents in Neurodegenerative Disease

While anthocyanins have proven to be promising therapeutic candidates in a preclinical context, it is important to note that the overall bioavailability of parent anthocyanins is very low despite their high degree of bioactivity. Indeed, the concentrations that are needed to achieve neuroprotection in cultured neurons far exceed physiological concentrations of anthocyanins observed in brain tissue from supplemented animals (Del Rio et al. 2010, Woodward et al. 2009). Furthermore, several reports have indicated that anthocyanins are rapidly and thoroughly metabolized in the gut such that very little of the parent compounds are able to be absorbed in their native state (Fleschhut et al. 2006, Woodward et al. 2009, Forester and Waterhouse, 2010). This has led to the hypothesis that anthocyanin metabolites are likely to mediate many of the biological effects observed following anthocyanin ingestion *in vivo.* Thus, it is of great import to

investigate the therapeutic properties of these metabolites in order to better understand their potential role in attenuating neurodegenerative disease

1.3.1 Metabolism, Absorption and Bioavailability of Anthocyanin Metabolites

Following consumption, anthocyanins are detected systemically in several forms, including sulfated, methylated, glucuronidated, and glycosylated conjugates (Gonthier et al. 2003, Manach et al. 2005). Additionally, metabolism by gut microflora has been shown to produce considerable amounts of phenolic acid and aldehyde metabolites that share many structural characteristics with their respective parent anthocyanins (Fleschhut et al. 2006; Woodward et al. 2009; Forester and Waterhouse, 2010). These metabolites are often more stable than parent anthocyanins supporting the idea that they are likely the bioactive components of an anthocyanin-rich diet (Keppler and Humpf, 2005, Kay et al. 2009).

It is generally accepted that anthocyanins and related polyphenols begin metabolism in the small intestine where glycoside linkages are hydrolyzed to produce the aglycon form (Day et al. 2000, Gee et al. 2000). Anthocyanins and aglycons that are not absorbed in the small intestine then pass on to the colon, where they are further metabolized by the resident microflora. Metabolism by gut microflora is perhaps the best characterized method by which anthocyanins are degraded and has been described by several studies. In this paradigm, anthocyanins incubated with intestinal microflora has been reported to lead to almost complete degradation of anthocyanins, resulting in the formation of phenolic acids and a universal aldehyde metabolite (Fig. 1.8; Fleschhut et al. 2006, Woodward et al. 2009, Forester and Waterhouse, 2010). The phenolic acids

derived from this process are the result of hydrolysis of the B-ring and largely retain their structure after separation from the anthocyanin skeleton (Fig 1.9). Phenolic acid metabolites can be further modified by glucuronidation; however, unaltered phenolic acids have been observed *in vivo* following anthocyanin ingestion (Woodward et al. 2011). In particular, the phenolic acid metabolite of cyanidin-based anthocyanins, protocatechuic acid (PCA), has been found in circulation, achieving a concentration eight times higher than that of parent anthocyanins in rat plasma (Azzini et al. 2010). Interestingly, PCA also appears to remain in tissue longer than its parent anthocyanin (Tsuda et al. 1999).

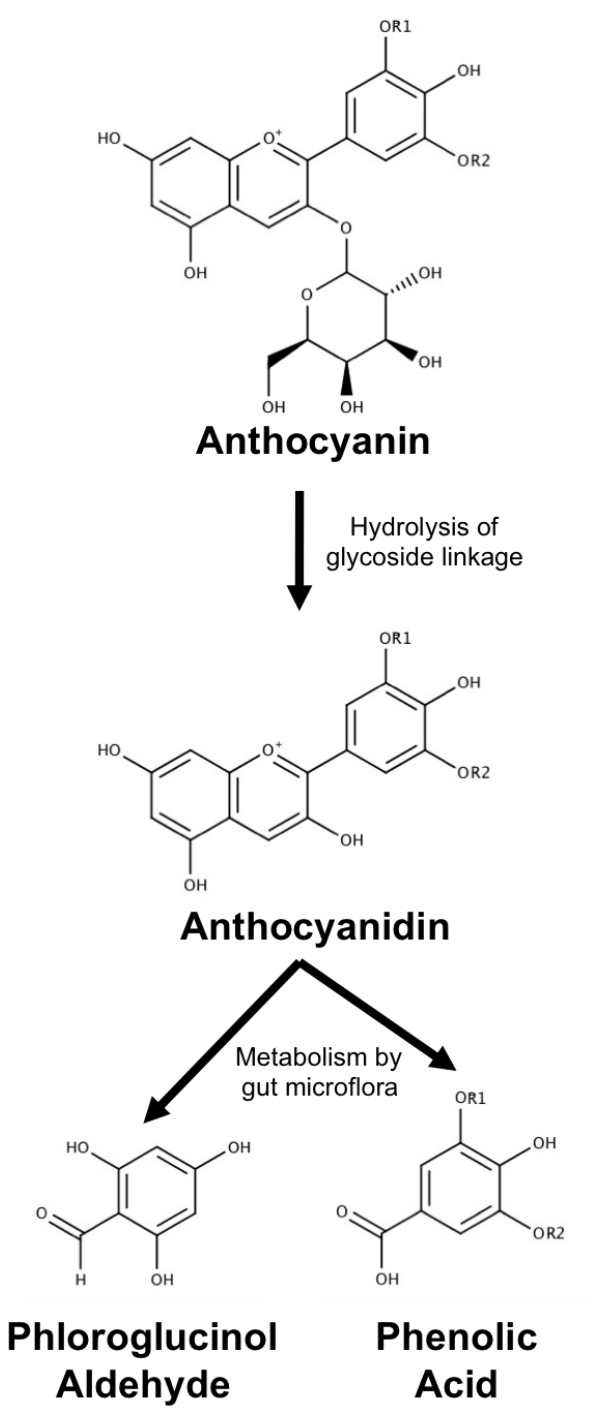

Figure 1.8. Metabolism of anthocyanins. A generic anthocyanin with a glucoside moiety is pictured. Parent anthocyanin species are first converted to an aglycon (anthocyanidin) form by hydrolysis of glycoside linkages in the small intestine. Upon entry into the large intestine, the anthocyanidin is further metabolized by gut microflora to produce a universal aldehyde metabolite, phloroglucinol aldehyde, and a phenolic acid that retains the structure of the B-ring of the parent anthocyanin.

A

Anthocyanin Base	Phenolic Acid Metabolite
Pelargonidin	4-Hydroxybenzoic Acid
Cyanidin	Protocatechuic Acid
Delphinidin	Gallic Acid
Peonidin	Vanillic Acid
Petunidin	3-O-Methylgallic Acid
Malvidin	Syringic Acid

B

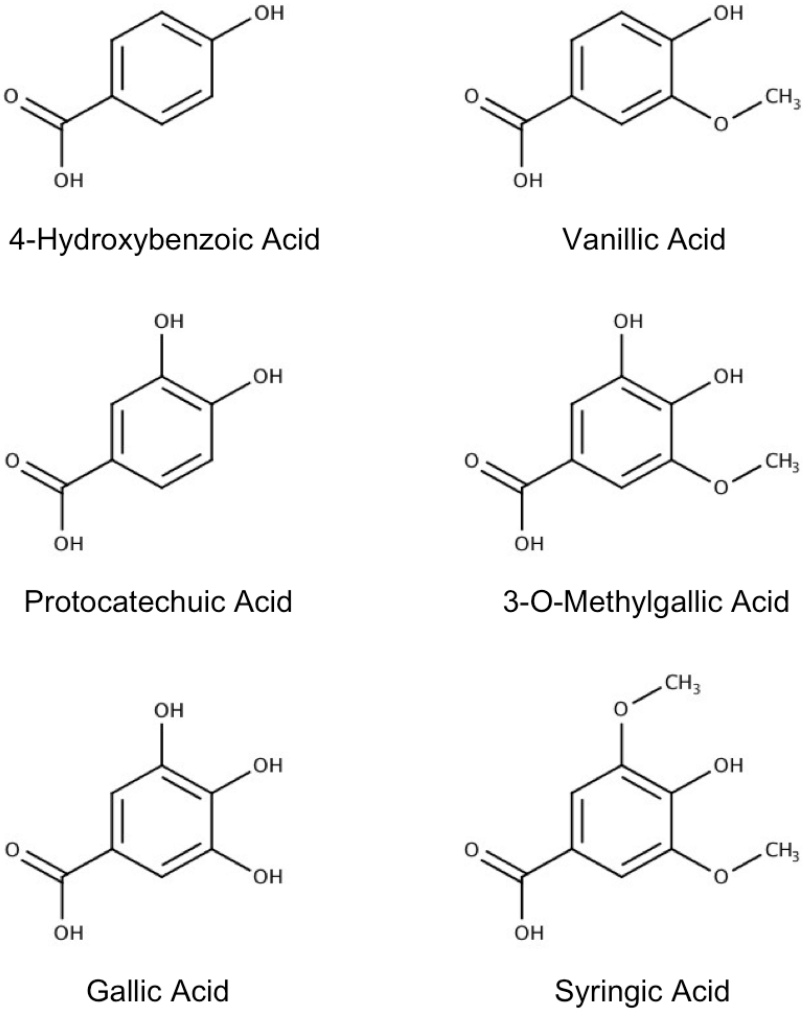

Figure 1.9. Common phenolic acid metabolites derived from anthocyanins. *A,* Common anthocyanin bases and their phenolic acid metabolites. *B,* Molecular structures of the six most common phenolic acid metabolites derived from anthocyanins.

While high circulating concentrations of some metabolites suggests that they may play a significant role in mediating the various health benefits of anthocyanin consumption, it is currently unknown if all anthocyanin metabolites are able to cross the BBB to be absorbed by relevant CNS tissues. However, a growing pool of evidence suggests that phenolic acids are indeed able to gain access to the CNS. One study has reported that PCA can be detected in cortical tissue from rats after oral administration of Danshen extract (Zhang et al. 2011). Gallic acid has also been detected in brain tissue following oral treatment with grape seed extract, and appears to accumulate in plasma and brain tissue with chronic dosing, strongly suggesting that these compounds are capable of crossing the BBB (Ferruzzi et al. 2009). Nevertheless, further exploration of this topic is required to assess the therapeutic efficacy of anthocyanin metabolites *in vivo* in order to characterize the bioavailability of these phenolic acids in the CNS.

1.3.2 *In Vitro* Neuroprotective Effects of Anthocyanin Metabolites

To date, studies assessing the neuroprotective effects of anthocyanin metabolites, particularly in primary cell cultures, are lacking; however the few studies that have been conducted show encouraging results for the use of these compounds in mitigating several factors associated with neuronal death in neurodegeneration. The large majority of these studies have examined the beneficial effects of the compounds PCA and gallic acid (GA), the phenolic acid metabolites of cyanidin- and delphinidin-based anthocyanins respectively (Fig. 1.9, Fleschhut et al. 2006, Woodward et al. 2009, Forester and Waterhouse, 2010).

Like its parent anthocyanins, PCA has been shown to have significant antioxidant capacity, which is believed to mediate some of its neuroprotective effects (Sroka and Cisowski, 2003). For example, treatment of neuronal cell lines with hydrogen peroxide induced a significant degree of cell death, which was entirely attenuated by treatment with PCA (An et al. 2006, Guan et al. 2006a, Shi et al. 2006, Tarozzi et al. 2007). This effect was likely mediated in part by the intrinsic free radical scavenging abilities of PCA; however it was also reported that PCA-treated PC12 cells challenged with hydrogen peroxide possessed higher levels of GSH and catalase activity, suggesting that this compound may also regulate intrinsic antioxidant defenses within the cell (Guan et al. 2006a). Similar results were observed in PC12 cells treated with the nitric oxide donor, sodium nitroprusside (SNP), with PCA preserving cellular viability to a significant degree (An et al. 2006).

PCA has also been examined for its ability to attenuate neuronal death induced by treatment with 1-methyl-4-phenylpyridinium (MPP+), the active metabolite of MPTP, which acts as a mitochondrial complex I inhibitor to induce neurotoxicity (Nicklas et al. 1985). These studies have indicated that PCA effectively protects PC12 cells from MPP+-induced toxicity by preventing mitochondrial dysfunction and elevating levels of antioxidant enzymes such as catalase, SOD1, and glutathione peroxidase, in addition to enhancing levels of the pro-survival protein, Bcl-2 (An et al. 2006, Guan et al. 2006b, Zhang et al. 2009). Similar results were observed when this study was repeated using rotenone, another inhibitor of complex I (Liu et al. 2008). GA and another anthocyanin metabolite, syringic acid, which is derived from malvidin-based anthocyanins, were also evaluated *in vitro* for their ability to attenuate 6-OHDA toxicity. This study found that

both GA and syringic acid were effective neuroprotective agents in this model, though the mechanism of protection was not well defined (Lu et al. 2006).

The impact of anthocyanin metabolites has also been evaluated for cellular models of neurotoxicity observed in Alzheimer's disease. PCA has been shown to mitigate the neurotoxic effects of amyloid beta in primary rat cortical neurons by attenuating increases in ROS and intracellular calcium levels, in addition to preventing glutamate release caused by this insult (Ban et al. 2007). Evidence has also accumulated suggesting that GA effectively defends neuronal cells from amyloid beta toxicity in a manner very similar to that of PCA, reducing accumulation of intracellular ROS and calcium, and preventing glutamate release (Bastianetto et al. 2006, Ban et al. 2008). Interestingly, regulation of calcium signaling has also been reported in one study utilizing GA to blunt kainate-induced excitotoxicity in PC12 cells. Indeed, GA treatment significantly attenuated increased calcium levels in addition to mitigating ROS accumulation and preventing the induction of pro-inflammatory enzymes such as COX-2 and p38-MAPK (Huang et al. 2012). Collectively, these studies indicate that PCA and GA may be effective treatments to regulate disruptions in calcium homeostasis observed in diseases like ALS.

GA has also been shown to ablate the neurotoxic effects of microglial inflammation induced by amyloid beta in a co-culture system utilizing either primary microglia or BV2 microglia and the neuro2A cell line (Kim et al. 2011). Treatment with GA significantly reduced production of pro-inflammatory cytokines, and induced acetylation of NF-κB, thereby decreasing inflammatory gene expression. This translated

into preservation of neuronal viability, suggesting that some anthocyanin metabolites may be effective anti-neuroinflammatory agents.

There is also a growing number of reports indicating that anthocyanin metabolites interfere with oligomerization of proteins such as amyloid beta and α-synuclein into toxic fibrils, which are characteristic of Alzheimer's and Parkinson's disease pathology, respectively (Table 1.1). GA was shown to interfere with amyloid beta fibril formation in primary rat hippocampal cells, which was thought to contribute to its protective effects against amyloid beta toxicity, though the mechanism of this effect is undefined (Bastianetto et al. 2006). In a similar fashion, both PCA and GA were found to inhibit the formation of toxic α-synuclein fibrils (Zhang et al. 2009, Liu et al. 2014). Though it is not clear how PCA carries out this function, GA was found to inhibit fibril formation by transient interaction with α-synuclein in its extended native structure, which prevented its collapse and subsequent formation of toxic fibrils (Liu et al. 2014). While further exploration is needed to determine if this effect can be reproduced with other anthocyanin metabolites, it is a promising finding for their use as therapeutic agents in neurodegeneration for which protein aggregation is a major contributing factor.

1.3.3 Anthocyanin Metabolites as Therapeutic Agents in Neurodegenerative Disease and Aging Models

Studies into the efficacy of anthocyanin metabolites for treating neurodegeneration have gained momentum in the past five years, and several reports have emerged describing the use of these compounds in preclinical disease models. While the data are sparse and focused almost exclusively around the *in vivo*

neuroprotective effects of PCA and GA, these initial reports have so far yielded encouraging findings. For example, the therapeutic potential of PCA was recently evaluated in the MPTP mouse model of Parkinson's disease. This study found that treatment with PCA prior to MPTP injection significantly ameliorated the detrimental effects of this insult, preventing loss of tyrosine hydroxylase-positive dopamine neurons in the substantia nigra and preserving levels of dopamine and its metabolites in striatum in comparison to mice receiving MPTP alone (Zhang et al. 2010). These observations correlated with improved motor function in rotarod testing. Similar results were reported in a study utilizing GA in the 6-OHDA rat model of Parkinson's disease. Rats that experienced 6-OHDA lesion displayed dramatic deficits in memory and learning performance, measured by passive avoidance testing, which correlated with increased lipid peroxidation and reduced antioxidant defenses in both hippocampus and striatum (Mansouri et al. 2013). These effects were significantly attenuated by GA treatment in a dose-dependent manner. These studies suggest that PCA and GA may be useful for the treatment of Parkinson's disease and other related disorders.

The therapeutic benefits of PCA and GA treatment were also explored in models of Alzheimer's disease and aging. In the APP/PS1 mutant mouse model of Alzheimer's disease, PCA treatment was found to reduce amyloid beta deposition in hippocampal tissue, in addition to reducing expression of APP, from which amyloid beta is generated (Song et al. 2014). PCA also reduced the presence of inflammatory markers in tissue homogenates from hippocampus and cerebral cortex including several inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8, while increasing production of pro-survival signaling factors such as brain-derived neurotrophic factor (BDNF). Mice that

received PCA treatment also displayed marked improvements in learning and memory tasks in comparison to untreated APP/PS1 mice (Song et al. 2014). These findings were corroborated in a study utilizing the D-galactose mouse model of aging, which reported similar anti-inflammatory effects following administration of PCA (Tsai and Yin, 2012). These included decreased levels of pro-inflammatory cytokines and reductions in COX-2 and NF-κB expression. Moreover, PCA treatment was also reported to reduce oxidative damage in the brains of treated mice. GA has been shown to have similar effects in mice treated with amyloid beta by intra-cerebro-ventricular injection. Amyloid beta injection caused significant impairments in learning and memory tasks in mice, in addition to enhancing pro-inflammatory markers including iNOS and COX-2 expression and production of IL-1β (Kim et al. 2011). NF-κB acetylation and nuclear translocation were also examined and found to be significantly enhanced in this model. Pre-treatment with GA, however, potently inhibited these effects, likely through suppression of NF-κB activity (Kim et al. 2011). These *in vivo* results support the assertion that phenolic acid metabolites of anthocyanins may be good candidates for the therapeutic treatment of Alzheimer's disease.

While the effects of anthocyanin metabolites have never been assessed in the context of ALS, the studies that have been conducted in other preclinical disease models indicate that these compounds may be effective therapeutic agents for ALS due to the common pathological features underlying these diseases. Nonetheless, further exploration is needed to fully understand the possible therapeutic value of phenolic acid metabolites for various neurodegenerative diseases.

1.4 Cellular Models for the Study of Neuroprotection and Neuroinflammation

The use of *in vitro* models is an essential step in the screening process for potential therapeutics. Cell culture models for neuroprotection and neuroinflammation provide relatively simple systems to assess the efficacy of a variety of compounds in a manner that is timely and cost-effective. Furthermore, cell culture models represent an easy way to gain insight into the mechanistic qualities of potential neuroprotective and anti-inflammatory compounds before introducing them into animal models of neurodegenerative disease. This makes them valuable tools for studying and identifying novel neuroprotective and anti-inflammatory compounds such as anthocyanins and their metabolites. Our approach uses two *in vitro* cell culture systems to assess the therapeutic potential of compounds in terms of their neuroprotective and anti-neuroinflammatory activity to inform subsequent *in vivo* analyses of promising therapeutic candidates in preclinical models of ALS. These systems are described in detail below.

1.4.1 An *In Vitro* Model of Neuroprotection: Cerebellar Granule Neurons

Cerebellar granule neurons (CGNs) are a widely used cell culture model to study the neuroprotective action of pharmacological, and more recently, nutraceutical compounds. Established over 40 years ago, CGN cultures represent a unique *in vitro* system to study neuronal death and survival, and have become one of the best characterized cell culture systems in neuroscience research. CGNs are among the most homogenous primary neuronal cultures, yielding purities of up to 95%, which is a desirable characteristic for studies attempting to define the mechanistic actions of a particular pharmacological or nutraceutical agent (Linseman et al. 2001). Furthermore,

treatment of CGNs with various insults (or cellular stressors) has been shown to recapitulate many of the individual molecular factors associated with neurodegeneration and cell death *in vivo*, including oxidative and nitrosative stress, excitotoxicity, dysregulation of protein homeostasis, and apoptosis. Indeed, treatment with hydrogen peroxide, 6-OHDA, an inducer of oxidative stress, or nitric oxide donors has been shown to induce apoptotic death of CGNs in a caspase-dependent manner (Leist et al. 1997, Wei et al. 2000, Lin et al. 2003, Noelker et al. 2005). Stimulation of CGNs with glutamate has also been shown to result in neuronal death due to excitotoxic mechanisms such as enhanced free-radical production and activation of calpains (Manev et al. 1991, Ciani et al. 1996). Additionally, CGNs have been shown to undergo apoptotic death in response to induction of ER stress by blocking transport of proteins from the ER to the Goli apparatus using Brefeldin A, and proteasome inhibition (Pasquini et al. 2000, Brewster et al. 2006, Ma and D'Mello, 2010). It is also notable that we have previously demonstrated that CGNs challenged with agents inducing mitochondrial oxidative stress displayed significant cell death consistent with apoptosis, which was almost entirely mitigated by treatment with pure anthocyanin compounds (Kelsey et al. 2011).

Thus, though many more studies exist demonstrating the value of CGNs in modeling different neuronal stressors, and the neuroprotective effects of pharmacological interventions, this small sampling of data indicate that CGNs are a versatile system for assessing the neuroprotective effects of anthocyanins against a variety of stressors (Fig 1.10). Moreover, data gained from this evaluation will provide valuable insight into the neuroprotective capacity and mechanism of action of these compounds to assess their suitability for further exploration in preclinical animals models of ALS.

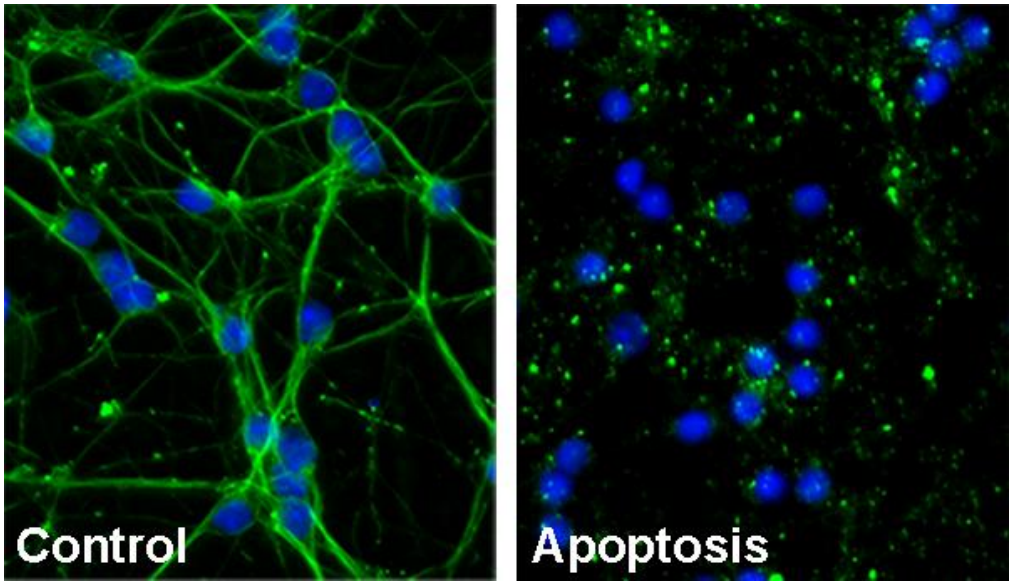

Figure 1.10. Cerebellar granule neurons as a model of cell death and survival. Cells were observed under normal culture conditions (Control) or in the presence of apoptotic stimuli (Apoptosis). Healthy control cells display large, round nuclei (blue) and elaborate tubulin networks (green) whereas cells that have undergone apoptosis possess condensed and fragmented nuclei and fragmented tubulin networks.

1.4.2 An *In Vitro* Model of Neuroinflammation: The BV2 Microglial Cell Line

As the primary mediators of the neuroinflammatory response, microglia play a critical role in the neurodegenerative process, and thus, many models of microglial inflammation have been developed. While the use of primary microglial cultures has been described for this purpose, the use of microglial cell lines represents a more cost-effective and timely way to screen for potential anti-inflammatory agents. The BV2 microglial cell line in particular is a widely used model of neuroinflammation as these cells retain many of the most important features of primary microglia (Horvath et al. 2008, Henn et al. 2009, Stansley et al. 2012). BV2 microglia were originally derived from primary mouse microglia immortalized by retroviral infection carrying the v-raf and v-myc oncogenes (Blasi et al. 1990). These cells were shown to maintain markers of a microglial phenotype, while lacking markers for other cell types such as glial fibrillary acidic protein (GFAP) and galactocerebroside, markers for astrocytes and oligodendrocytes respectively (Blasi et al. 1990). Furthermore, these cells behave in the same way as primary microglia and maintain the ability to mount an inflammatory response in the presence of LPS and interferon-γ, and perform phagocytosis (Blasi et al. 1990, Kopec and Carroll, 1998). Indeed, 90% of the genes activated by LPS stimulation in primary microglia are also activated in BV2 microglia, albeit to a lesser extent (Henn et al. 2009). Moreover, BV2 microglia retain the ability to secrete pro-inflammatory factors such as cytokines and nitric oxide, though overall levels are lower than those observed in primary microglia, and their use in co-culture systems with primary neurons has been shown to induce apoptotic neuronal death upon microglial activation in a

manner similar to that hypothesizied to occur *in vivo* during neurodegeneration (Bi et al. 2014, Henn et al. 2009, Horvath et al. 2008).

The use of BV2 microglia to evaluate the anti-neuroinflammatory effects of anthocyanin-rich extracts and at least one anthocyanin metabolite has recently been described and treatment with these substances was shown to have a positive impact on microglial inflammation (Lau et al. 2007, Kim et al. 2011, Poulose et al. 2012, Carey et al. 2013, Jeong et al. 2013). With this in mind, BV2 microglia are a viable system to further evaluate the therapeutic benefits of anthocyanins and their metabolites *in vitro* before considering the use of these compounds in preclinical models of ALS (Fig. 1.11).

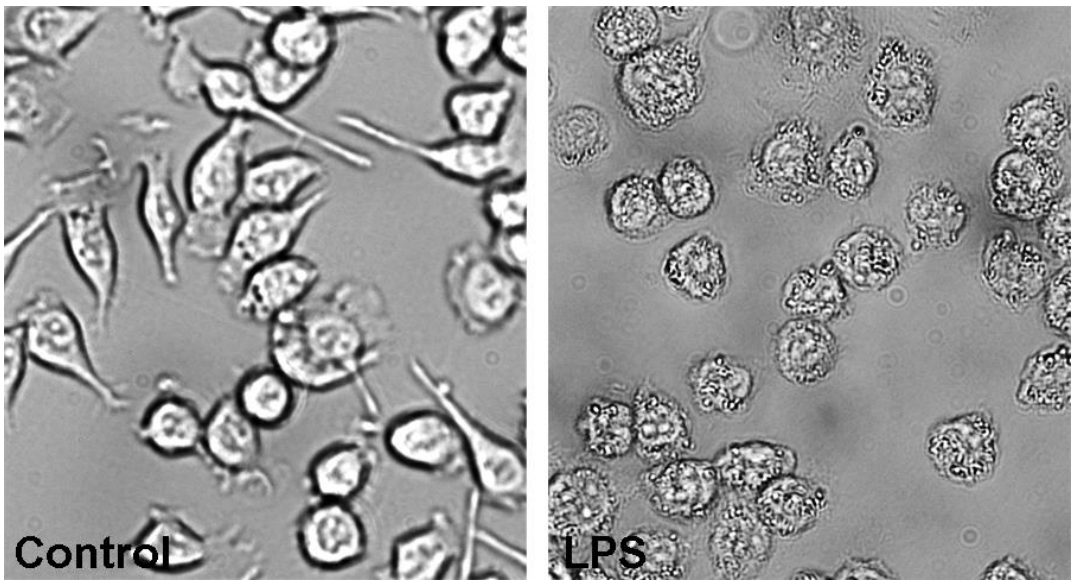

Figure 1.11. BV2 microglia as a model of neuroinflammation. BV2 cells were observed under normal culture conditions (Control) or after treatment with lipopolysaccharide (LPS) for 24h. Control cells possess a spindle-shaped morphology characterized by long processes extending from the cell body. Cells treated with LPS display a rounded, amoeboid morphology, and a loss of processes extending from the cell body, characteristic of inflammatory microglia.

1.5 Preclinical Assessment of Therapeutic Agents: The G93A mutant hSOD1 Mouse Model of ALS

Mutations in SOD1 were first described by Rosen et al. in 1993 as the first genetic causes of autosomal dominant familial ALS. Shortly following this discovery, the first transgenic mutant mouse model of ALS, the hSOD1^{G93A} mouse, was developed in an effort to better our understanding of ALS disease course (Gurney et al. 1994). This mouse model has become the most widely used preclinical model of ALS, and a majority of what we currently understand about ALS pathophysiology and the treatment of this disease has been derived from this model.

Mice overexpressing the hSOD1^{G93A} transgene faithfully recapitulate many clinical manifestations of ALS disease progression. These mice experience early disease onset at approximately three to four months of age depending on the background of the strain, which is characterized as hind limb weakness, measured by the inability of mice to splay their hind limbs when suspended by the tail, and weight loss (Fig. 1.12; Gurney et al. 1994, Ludolph et al. 2007, Scott et al. 2008). Disease progression is aggressive in this model, occurring over the course of approximately three to four weeks during which time mice experience continued loss of strength in the hind limbs, significant loss of body weight, muscle atrophy, and paralysis until end stage of disease is reached, defined as the point at which a mouse placed on its side can no longer right itself to a sternal position (Ludolph et al. 2007, Scott et al. 2008).

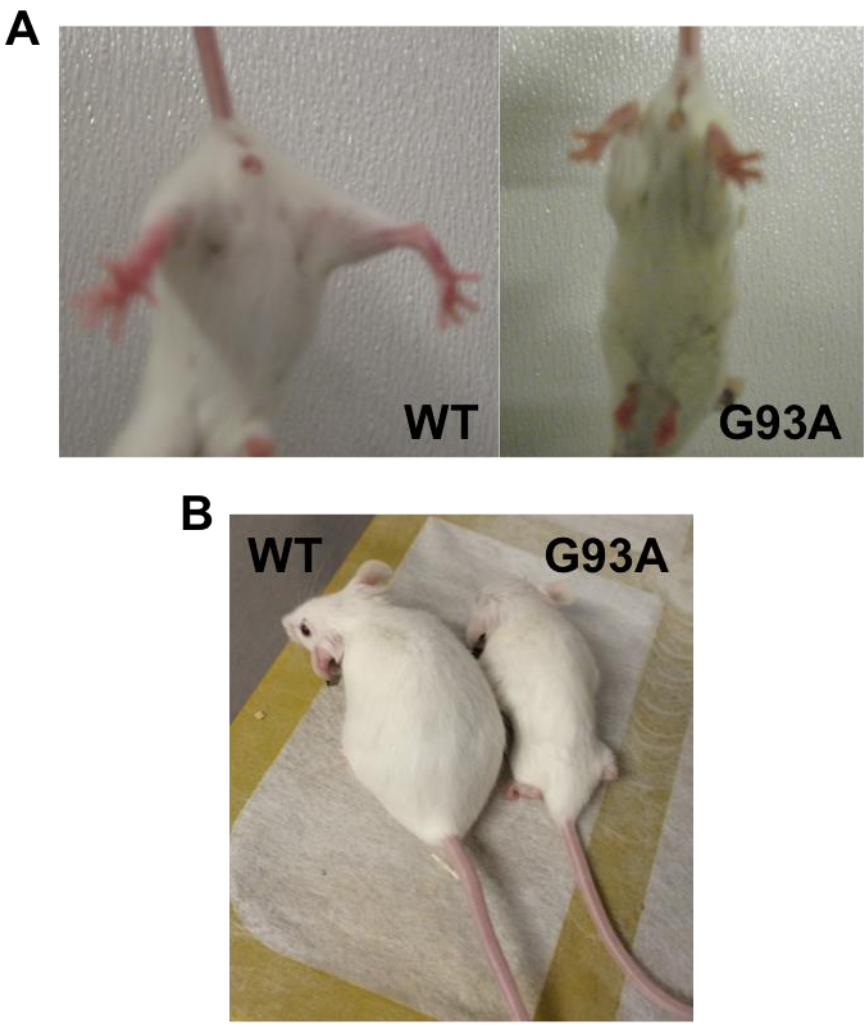

Figure 1.12. Clinical indices of disease in the hSOD1^{G93A} mouse model of ALS. *A,* Transgenic hSOD1^{G93A} mice display significant weakness in hind limb muscles measured by leg splay. Non-transgenic wild type (WT) mice splay their hind limbs when suspended by their tail whereas hSOD1^{G93A} (G93A) mice cannot splay their hind limbs due to muscle weakness and paralysis. *B,* Transgenic hSOD1^{G93A} mice display muscle atrophy and weight loss. WT mice show healthy weight and muscle mass, whereas hSOD1^{G93A} mice show marked reductions in body weight and significant muscle atrophy, particularly in the hind limbs.

In addition to clinical symptoms of ALS, hSOD1^{G93A} mice display several histopathological markers of ALS disease pathophysiology. Among these, the most notable is rapid and extensive degeneration of motor neurons (particularly spinal motor neurons) resulting in motor neuron axonopathies, and eventual death (Gurney et al. 1994, Dal Canto and Gurney 1994). As loss of motor neurons is a defining characteristic of ALS disease pathology, this represents solid evidence that mice harboring the mSOD^{G93A} transgene do develop ALS-like disease. Furthermore, hSOD^{G93A} mice also develop mitochondrial dysfunction and oxidative stress, markers of excitotoxicity, markers of ER stress, reactive gliosis, and loss of functional neuromuscular junctions in a manner that appears similar to that experienced by ALS patients (Dal Canto and Gurney, 1994, Hall et al. 1998a, Hall et al. 1998b, Kong and Xu, 1998, Mattiazzi et al. 2002, Saxena et al. 2009, Wootz et al. 2010, Milanese et al. 2011, Giribaldi et al. 2013). These mice also develop SOD1-positive aggregates consistent with histopathological data from familial ALS patient tissue (Turner et al. 2013). As discussed above, all of these features have been observed in patients with both sporadic and familial forms of ALS, suggesting that findings in the hSOD1^{G93A} mouse model could be applicable to both forms of the disease observed in ALS patients.

Because the hSOD1^{G93A} mouse model of ALS appears to share many pathological features with human disease and displays a well-characterized disease course, it has become the model of choice for rapid preclinical screening and characterization of numerous proposed therapies to treat ALS. In recent years, interest in nutraceutical treatments for ALS has grown owing largely to the observation that many nutraceutical compounds display impressive antioxidant abilities, and several notable studies have

been conducted examining their efficacy as therapeutic agents. Vitamin E is arguably one of the most studied nutraceuticals yet examined for the treatment of ALS. Aimed at reducing oxidative damage, particularly in lipid membranes, presymptomatic vitamin E supplementation in the hSOD^{G93A} mouse significantly delayed disease onset, though no extension in survival of treated mice was noted (Gurney et al. 1996). Subsequent clinical trials with vitamin E in ALS patients have been conducted, though the efficacy for this nutraceutical in treating ALS remains unclear (Butterfield et al. 2002). We recently obtained similar results using a non-denatured whey protein supplement, Immunocal®, which is known to boost GSH levels *in vivo* (Ross et al. 2014). Transgenic hSOD1^{G93A} mice treated presymptomatically with this supplement displayed a significant delay in disease onset and preservation of skeletal muscle function, although no extension in survival was observed (Ross et al. 2014). This correlated with preservation of GSH levels in blood and spinal cord tissue isolated from treated hSOD1^{G93A} mice, though Immunocal® treatment was not able to rescue GSH deficits specifically in the mitochondrial compartment of spinal cord tissue. Though modest therapeutic benefits were observed in both of these studies, they highlight the use of the hSOD1^{G93A} mouse as a viable and sensitive model in which to test the mechanism and efficacy of novel therapeutic candidates for ALS treatment. Therefore, the hSOD1^{G93A} mouse model of ALS represents a powerful tool to evaluate the therapeutic value of anthocyanins and their metabolites *in vivo*.

CHAPTER TWO: CHEMICAL BASIS FOR THE DISPARATE NEUROPROTECTIVE EFFECTS OF THE ANTHOCYANINS, CALLISTEPHIN AND KUROMANIN, AGAINST NITROSATIVE STRESS

2.1 Abstract

Oxidative and nitrosative stress are major factors in neuronal cell death underlying neurodegenerative disease. Thus, supplementation of antioxidant defenses may be an effective therapeutic strategy for diseases such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease. In this regard, treatment with nutraceutical antioxidants has garnered increasing attention; however, the differential neuroprotective effects of structurally similar nutraceuticals, which may affect their suitability as therapeutic agents, has not been directly examined. In this study we compare the ability of two anthocyanins, callistephin (pelargonidin-O-3-glucoside) and kuromanin (cyanidin-O-3-glucoside) to protect cerebellar granule neurons from damage induced by either oxidative or nitrosative stress. These anthocyanins differ by the presence of a single additional hydroxyl group on the B-ring of kuromanin, forming a catechol moiety. While both compounds protected neurons from oxidative stress induced by glutamate excitotoxicity, a stark contrast was observed under conditions of nitrosative stress. Only kuromanin displayed the capacity to defend neurons from nitric oxide (NO)-induced apoptosis. This protective effect was blocked by addition of Cu, Zn-superoxide

dismutase, indicating that the neuroprotective mechanism is superoxide dependent. Based on these observations, we suggest a unique mechanism by which slight structural variances, specifically the absence or presence of a catechol moiety, lend kuromanin the unique ability to generate superoxide, which acts as a scavenger of NO. These findings indicate that kuromanin and compounds that share similar chemical characteristics may be more effective therapeutic agents for treating neurodegenerative diseases than callistephin and related (non-catechol) compounds.

2.2 Introduction

Conditions of oxidative and nitrosative stress are well documented factors contributing to the pathophysiology of a number of neurodegenerative diseases (Calabrese et al. 2000, Lin and Beal 2006, Gu et al. 2010, Radi et al. 2014). In recent years, it has become apparent that these conditions arise largely as a consequence of diverse initiating mechanisms, including excitotoxicity, mitochondrial dysfunction, and neuroinflammation, which produce large quantities of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Accumulation of ROS, such as superoxide, hydrogen peroxide, and hydroxyl radicals, or RNS, such as nitric oxide and peroxynitrite, results in significant damage to vital cellular components, culminating in neuronal cell death. It is therefore unsurprising that strategies aimed at mitigating the damaging effects of ROS and RNS are being actively pursued as potential therapeutic treatments for a number of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS). In this respect, flavonoids, naturally occurring

compounds found in a number of highly consumed fruits and vegetables, are promising therapeutic candidates due to their impressive antioxidant, anti-inflammatory and anti-apoptotic properties, which make them well suited to combat multiple facets of the neurodegenerative process (Ishikawa et al. 2000, Hirano et al. 2001, Schroeter et al. 2001, Kwon et al. 2004, Kumazawa et al. 2006, Hamalaninen et al. 2007, Vauzour et al. 2007, Vauzour et al. 2008).

In particular, anthocyanins, a class of flavonoid compounds responsible for the brilliant red, blue, and purple coloration of many fruits, vegetables and flowers, have garnered significant attention as potent antioxidants and anti-inflammatory agents. Their positive effects on a myriad of health conditions, such as heart disease and cancer, have been extensively documented (reviewed by Wang and Stoner 2008, Wallace 2011, Ross et al. 2012). However, while promising, the use of these compounds in the context of neurodegenerative disease is novel and warrants further exploration.

While several studies have explored the benefits of anthocyanins in both *in vitro* and *in vivo* models of neurodegeneration, neuroinflammation, and aging (Poulose et al. 2012, Ross et al. 2012, Carey et al. 2013, Jeong et al. 2013, Ross et al. 2013), interpreting the results of these studies is often complicated by the use of anthocyanin-enriched extracts, which contain multiple anthocyanins and small amounts of other (poly)phenolic compounds. While such studies have been invaluable as a starting point for identifying anthocyanins as new drug candidates, the presence of multiple compounds within a single extract severely limits the ability of investigators to discern which compounds are ultimately responsible for any observed health benefits, and eliminates the possibility of

dissecting the mechanism of action of any particular compound. Interpretation of results is further confounded by the possibility that any positive effects observed after treating with an anthocyanin-enriched extract may actually be due to synergism between several unique compounds, rather than the activity of a single agent. Indeed, a recent study by Carey et al. (2013) compared the anti-neuroinflammatory effects of two pure anthocyanin species to an extract derived from blueberries using the BV2 microglial cell line. Their data revealed that a much higher concentration of pure anthocyanins was required to achieve significant reductions in inflammatory markers when compared to the blueberry extract, suggesting a high degree of synergism between its constituents.

In this regard, conclusions drawn using anthocyanin-enriched extracts alone are restricted at best to a particular family of compounds, which contains several members, or at worst, to the use of a particular extract itself. However, despite these limitations, studies using pure anthocyanin species in models of neurodegeneration and neuroprotection are few in number, and almost exclusively limited to *in vitro* assessment (Tarozzi et al. 2007, Tarozzi et al. 2008, Kim et al. 2009, Tarozzi et al. 2010, Kelsey et al. 2011, Shih et al. 2011). Indeed, to the best of our knowledge, only two studies have been conducted *in vivo,* one using the pure anthocyanidin, pelargonidin, in a 6-hydroxydopamine lesioned rat model of hemi-Parkinsonism, and one using cyanidin-*O*-3-glucoside to preserve spatial memory in rats treated with beta-amyloid peptide (Roghani et al. 2010, Qin et al. 2013). Furthermore only one *in vitro* study previously conducted by our lab with pure anthocyanins has compared the neuroprotective effects of distinct

anthocyanin species (Kelsey et al. 2011). However, this study was limited to the evaluation of anthocyanins only in the context of mitochondrial oxidative stress.

While our previous work indicated that the two pure anthocyanins, callistephin (pelargonidin-*O*-3-glucoside) and kuromanin (cyanidin-*O*-3-glucoside), protect primary neurons equally from the Bcl-2 inhibitor and mitochondrial glutathione depleting agent, HA14-1, reports comparing the properties of different anthocyanins and related flavonoids in non-neuronal systems have indicated that subtle differences in molecular structure, particularly the number of hydroxyl groups located on the B-ring, have measurable effects on the ability to interact with lipid membranes, absorption, and free radical scavenging activities (Yi et al. 2006, Brown and Kelly, 2007, Kelsey et al. 2011). With this in mind, we sought to determine if structurally similar, yet distinct anthocyanin species also differ in their ability to confer neuroprotection against a more diverse array of insults related to neurodegenerative processes, such as excitotoxicity and nitric oxide toxicity. Our results revealed a striking contrast in the neuroprotective abilities of the anthocyanins callistephin and kuromanin against nitric oxide-induced nitrosative stress, with only kuromanin significantly preserving cell viability. As these compounds are structurally similar, differing only by the presence of a single hydroxyl group on the B-ring of kuromanin (Fig. 2.1), we determined that slight alterations in molecular structure can have a profound effect on neuroprotective capacity under conditions of nitrosative stress. Here, we propose a novel neuroprotective mechanism by which anthocyanins containing a catechol moiety on their B-ring, such as kuromanin, are able to effectively scavenge and detoxify harmful nitric oxide radicals.

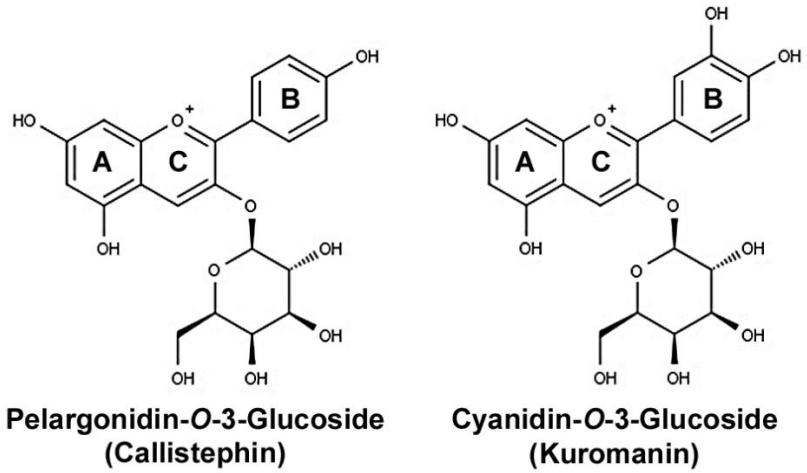

Figure 2.1. Molecular structures of callistephin (left) and kuromanin (right). Note the catechol moiety on the B-ring of kuromanin.

2.3 Methods

2.3.1 Reagents

Callistephin chloride (≥ 97% purity), kuromanin chloride (≥ 95% purity), glutamic acid, glycine, potassium chloride, Hoechst 33258, paraformaldehyde, polyethyleneglycol-conjugated superoxide dismutase 1 (PEG-SOD), Cu, Zn-superoxide dismutase (SOD1) from bovine erythrocytes, DT diaphorase (quinone oxidoreductase 1; QR1), quininone oxidoreductase 2 (QR2), tween 20, and monoclonal antibody against β-tubulin were purchased from Sigma-Aldrich (St. Louis, MO). Sodium nitroprusside (SNP) was obtained from Calbiochem (San Diego, CA). 2-Methyl-6-(4-Methoxyphenyl) -3,7-Dihydroimidazo[1, 2-A]pyrazin-O-3ne, Hydrochloride (MCLA) was purchased from Molecular Probes (Grand Island, NY). Basal Medium Eagle's solution, L-glutamine solution, penicillin/streptomycin solution, fetal bovine serum (FBS), and lipofectamine 2000 transfection reagent was purchased from Invitrogen (Grand Island, NY). Anti-mouse FITC-conjugated secondary antibody was obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Nitric Oxide Assay kit (EMSNOTOT) was obtained from Thermo Scientific (Rockford, IL). MTT assay kit was purchased from Bioassays Systems (Hayward, CA). Nutri-Fruit freeze-dried strawberry and blackberry powders were purchased from the Scenic Fruit Company (Gresham, OR).

2.3.2 Extraction and HPLC Analysis of Freeze-Dried Fruit Powders

Anthocyanin enriched extracts from strawberry (SAE) and blackberry (BAE) were prepared using commercially available freeze-dried fruit powders. Extracts were

prepared as described by Rodriguez-Saona and Wrolstad (2001) using acidified methanol. Fifty grams of freeze-dried fruit powder was placed in a large flask and macerated with acidified methanol (0.01% HCl) for 1 hour at room temperature to create a crude extract of phenolic species. Methanol containing the extracted (poly)phenols was then collected by vacuum filtration using Whatman no. 1 filter paper. The remaining powder collected in a Buchner funnel, was then re-extracted with acidified methanol until a faint colored extract was obtained. The crude extract was pooled together and boiled under vacuum at a temperature of approximately 40°C to remove the methanol until the extract had become viscous. This extract was then purified for anthocyanins using a C18 column prepared with acidified water (0.01% HCl). The extract was loaded onto the column then washed sequentially with one column volume each of acidified water and ethyl acetate, and then eluted into a collection flask using acidified methanol (0.01% HCl). This extract, containing purified anthocyanins, was then boiled once more under vacuum at a temperature of approximately 40°C until a highly viscous and strongly pigmented solution was obtained.

HPLC analyses were conducted using HPLC-UV/Vis with an Agilent model HP1100 series II with programmable diode array detector (Avondale, PA). Separation of anthocyanins was carried out using a C18 bonded silica column (3µm, 3 x 150mm) from Dionex Inc. (Sunnyvale, CA). Mobile phases consisted of A, 0.1% trifluoroacetic acid (TFA) in water, and B, 70% acetonitrile in water, which were combined in a step-wise gradient over the course of the separation. Gradient conditions were as follows: initial conditions were composed of 90% mobile phase A and 10% mobile phase B; from time

5-10 minutes, the gradient changed to 30% mobile phase B; from 14.5-16 minutes, the gradient changed to 50% mobile phase B; from 20-21 minutes, the gradient was returned to 10% mobile phase B. Analysis was carried out on 10µL injections at 25˚C using a flow rate of 0.6mL/min, and anthocyanins were detected at 520nm over the course of 25 minutes with a post run time of 5 minutes to clear and equilibrate the column for subsequent runs.

The concentration of the primary anthocyanin constituent in both SAE and BAE was determined by creating a standard curve from commercially available anthocyanin standards for both callistephin chloride and kuromanin chloride. For each anthocyanin, a 1mg/mL solution was prepared initially in 0.1% TFA in water, and diluted to 3/5, 2/5, 1/5, and 1/10 of the original concentration. Standards were injected individually and run under the HPLC conditions described above to create linear curves ($R^2 \geq 0.999$) for each anthocyanin species.

2.3.3 Cell Culture

Cerebellar granule neurons (CGNs) were isolated as previously described (Linseman et al. 2001) from seven day-old Sprague-Dawley rat pups, including both sexes. Cells were plated on poly-L-lysine coated six-well plates (35mm-diameter), with a density of approximately $4x10^6$ cells/well in Basal Medium Eagle's supplemented with 25mM potassium chloride, 10% FBS, 2mM L-glutamine, and penicillin/streptomycin (100U/mL/100µg/mL). Twenty-four hours after CGNs were plated, 10µM cytosine arabinoside was added to the culture medium to inhibit the growth of non-neuronal cells.

Granule neurons were subsequently incubated at 37˚C in 10% CO_2 for six to seven days in culture prior to experimentation. At this point, cultures were ~95% pure for CGNs.

Neuro2A cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2mM L-glutamine, and penicillin/streptomycin (100U/mL/100µg/mL) at 37˚C in 10% CO_2. Cells were plated in 6-well plates (35mm-diameter), and allowed to reach approximately 80% confluency before transfection and treatment.

2.3.4 Transfection of Neuro2A cells

Neuro2A cells were plated at 50% confluency and transfected the following day when the cells had reached approximately 80% confluency using the Lipofectamine 2000 reagent according to the manufacturer's instructions. Briefly, culture medium was removed and replaced with OptiMEM transfection medium. Plasmids encoding either enhanced green fluorescent protein (EGFP) or an EGFP-Cu, Zn-superoxide dismutase (SOD1) fusion construct were incubated for 20 minutes in OptiMEM containing Lipofectamine 2000 at a dilution of 1:1000. This mixture was then added to culture dishes containing Neuro2A cells in OptiMEM transfection medium and cells were allowed to incubate for 6 hours at a temperature of 37˚C. Following the 6 hour incubation, the transfection medium was removed and replaced with culture medium at which time cells were treated as described below.

2.3.5 Treatment of cell cultures

Cell culture medium was removed and replaced with serum free medium in order to avoid any potential protective effects of the serum that might limit apoptosis. Cells were then treated with either 100µM glutamate (final solution contained 100µM glutamic acid and 10µM glycine co-factor) or 100µM SNP alone or in combination with 0.15% SAE, 0.25% BAE, 100µM callistephin, or 100µM kuromanin for 24 hours prior to quantification of apoptosis. For those experiments utilizing Cu, Zn-superoxide dismutase (SOD1), cells were treated as described above with the addition of either PEG-SOD or unconjugated SOD1 at a concentration of 30U/mL unless otherwise indicated. For all experiments, an untreated control was used for comparison in assaying cell death.

2.3.6 MTT Assay

An MTT assay kit was used to determine the viability of cells treated with SAE and BAE per the manufacturer's instructions. Briefly, MTT reagent was added to each well of a 6-well culture dish following treatment with SAE and BAE at a final concentration of 2mM, and cells were then incubated at 37˚C for 4 hours. Following this incubation, the purple formazan precipitate was solubilized using 2mL of dimethyl sulfoxide (DMSO) by gently rocking the culture dishes for 1 hour at room temperature until all of the precipitate had dissolved. This solution was then transferred to a 96-well plate, and the absorbance of the sample was determined at 570nm, and expressed as a percentage of the untreated control.

2.3.7 Immunocytochemistry and Assay of Apoptosis

Following treatment, CGNs were washed with phosphate buffered saline (PBS; pH=7.4) and fixed for one hour in 4% paraformaldehyde. Cells were then washed with PBS and incubated in blocking solution containing 5% bovine serum albumin (BSA) in 0.2% triton-X in PBS for 1 hour at room temperature. This was followed by overnight incubation at 4˚C with primary antibody against β-tubulin, prepared in a 1:200 dilution in 2% BSA in 0.2% triton-X in PBS. The following day, CGNs were washed, and then incubated for 1 hour at room temperature with FITC-conjugated secondary antibody prepared as a 1:250 dilution in 2% BSA in 0.2% triton-X in PBS with Hoechst stain at 10mg/mL. Neuro2A cells were similarly washed with PBS following transfection and treatment, then fixed for one hour at room temperature. These cells were then stained with Hoechst to visualize nuclear morphology. All cells were imaged using a Zeiss Axiovert-200M epi-fluorescence microscope. Five images per well were captured to assay apoptosis, with either duplicate or triplicate wells for each treatment comprising one experiment. Cells were counted and scored as either living or apoptotic based on nuclear morphology using images showing decolorized Hoechst fluorescence. Cells having condensed or fragmented nuclei were scored as apoptotic, and a minimum of 50 cells per treatment per experiment were scored overall.

2.3.8 Nitric Oxide Assay

Nitric oxide assay was performed using a Nitric Oxide Assay kit from Thermo Fisher Scientific (Rockford, IL) by the Griess method per the manufacturer's instructions. This assay was used to determine the total amount of both nitrate and nitrite ions in

solution, which are generated as the major degradation products of nitric oxide. Samples were prepared in sealed 1.5-mL mirocentrifuge tubes in the absence of cells in serum free medium containing 25mM KCl, and either 100µM callistephin or kuromanin ± 100µM SNP. Samples containing only medium, or SNP only in medium were prepared in parallel for comparison. All samples were placed in an Eppendorf thermomixer, and allowed to incubate overnight at 37˚C and 300 rpm before assaying nitric oxide concentration. Since the culture medium does not contain nitrates or nitrites, readings from a sample containing culture medium only were subtracted as background from the total absorbance for all of the remaining samples before calculating the concentration of nitric oxide in solution. Absorbance for samples containing callistephin and kuromanin without SNP were similarly subtracted from samples containing both anthocyanins and SNP in order to correct for the absorbance of these compounds when assaying for nitric oxide production.

2.3.9 Superoxide Assay

Superoxide production was assayed using MCLA luminescence as an indicator for the presence of superoxide radical. Samples were prepared in a total volume of 1mL in PBS containing 100µM MCLA and either 100µM kuromanin alone, or in combination with 5U/mL QR1 or QR2. Reactions containing QR1 also contained 200µM NADH to serve as a cofactor, while reactions containing QR2 also contained 200µM of 1-methyl-1, 4-dihydronicotinamide cofactor. A 30U/mL SOD1 aliquot was added to one sample for each experiment containing kuromanin and QR2 as a control to confirm that MCLA luminescence was due to superoxide, and not production of another reactive radical

species. Luminescence was recorded every 15 seconds for a period of 8 minutes. A baseline was recorded for the first 4 minutes of each experiment at which time kuromanin, or kuromanin in combination with SOD1 was added to the reaction mixture. For those experiments in which the effects of QR1 or QR2 were assessed, these enzymes were added 1 minute after the addition of kuromanin or kuromanin in combination with SOD1. Luminescence was measured using a TD-20/20 model luminometer from Turner Designs (Sunnyvale, CA).

2.3.10 Data Analysis

Each experiment was performed using either duplicate or triplicate wells for each treatment, with each experiment being performed at least three times. Data represent the means ± standard error of the mean (SEM) for the total number (n) of experiments carried out. All data, unless otherwise noted, were analyzed using a one-way analysis of variance (ANOVA) with a *post hoc* Tukey's test. Data collected for the MCLA superoxide assay were analyzed using two-way ANOVA for repeated measures with a *post hoc* Bonferroni's test to compare all groups. A p-value <0.05 was considered statistically significant.

2.4 Results

2.4.1 Anthocyanin-Enriched Extracts from Strawberries and Blackberries Display Differential Neuroprotective Effects Against Oxidative and Nitrosative Stress

Anthocyanin-enriched extracts were prepared from freeze-dried strawberry and blackberry powder and characterized by HPLC. Anthocyanins were detected at a wavelength of 520 nm and the identity of the primary anthocyanin constituent of each extract was determined by comparison to pure anthocyanin standards (Fig. 2.2).

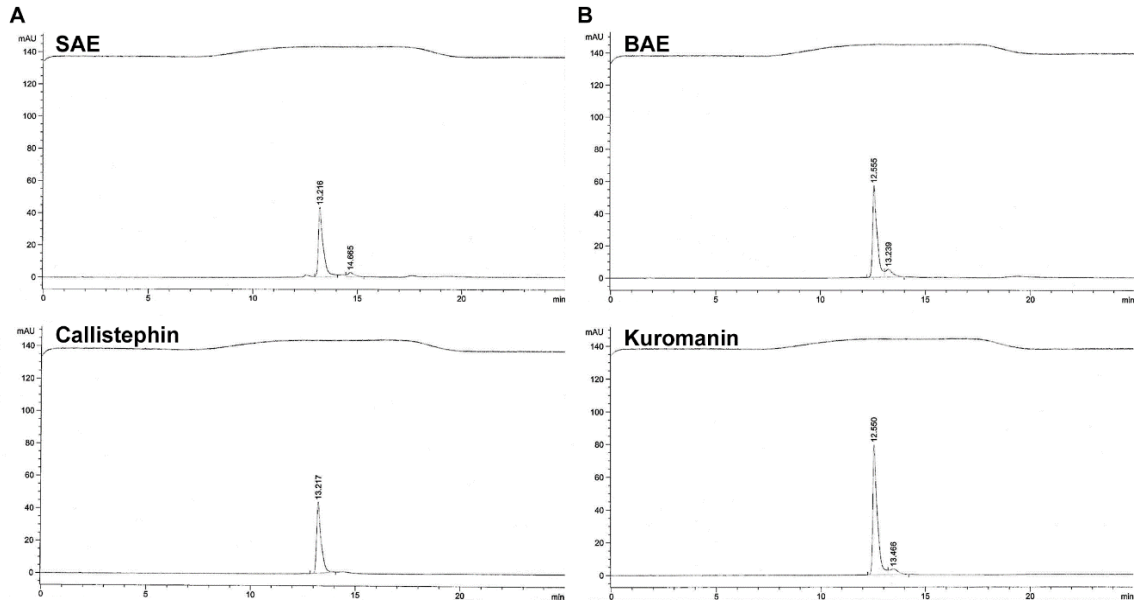

Figure 2.2. Representative HPLC chromatograms of anthocyanin-enriched extracts from strawberry (SAE) and blackberry (BAE). *A,* Purified anthocyanin extract from freeze-dried strawberry powder was analyzed by HPLC with UV/Vis detection at 520nm (top panel) and compared to a pure callistephin standard (bottom panel) for peak identification of the major anthocyanin constituent. Callistephin appears at rt=13.22min for SAE and rt=13.22min for the pure standard; *B,* Purified anthocyanin extract from freeze-dried blackberry powder was analyzed by HPLC with UV/Vis detection at 520nm (top panel) and compared to a pure kuromanin standard (bottom panel) for peak identification of the major anthocyanin constituent. Kuromanin appears at rt=12.56min for BAE and rt=12.55min for the pure standard. rt: retention time.

By this method, it was determined that the primary anthocyanin constituent of strawberry anthocyanin extract (SAE) was callistephin, which made up 91% of the enriched extract, while blackberry anthocyanin extract (BAE) was composed primarily of kuromanin, which made up approximately 87% of the enriched extract (Table 2.1). Once the identity of the primary anthocyanin constituents of each extract had been determined, the concentration of this component was determined for both SAE and BAE by comparison to a standard curve created using varying concentrations of either callistephin or kuromanin accordingly. For SAE, callistephin was determined to have a concentration of 5mM. For BAE, the concentration of kuromanin was calculated to be 3mM (Table 2.1). In experiments using these extracts, the concentrations of SAE and BAE were adjusted on a percentage basis to yield equivalent concentrations (7.5μM) of their respective primary anthocyanin constituents.

Table 2.1. Composition and concentration of primary anthocyanin constituents in Strawberry Anthocyanin Extract (SAE) and Blackberry Anthocyanin Extract (BAE).

Source of Anthocyanin Extract	Primary Anthocyanin Constituent	Percent Composition of Primary Anthocyanin	Concentration of Primary Anthocyanin in Extract	Percentage of SAE or BAE in Culture	Final Anthocyanin Concentration in Culture
Strawberry	Callistephin	91%	5mM	0.15%	7.5µM
Blackberry	Kuromanin	87%	3mM	0.25%	7.5µM

As anthocyanins are known to be potent antioxidants, we next characterized the neuroprotective profile of the two extracts against distinct neurotoxic insults. Excitotoxicity, during which neurons become over stimulated by glutamate signaling, leading to massive calcium influx, and subsequent activation of multiple cell death processes, was used as a model of oxidative stress as increased production of ROS is a hallmark of this process (Luetjens et al. 2000). Moreover, neuronal cell death associated with excitotoxicity is thought to play a role in several neurodegenerative diseases such as ALS, Parkinson's disease and Alzheimer's disease, highlighting the relevance of this insult for screening potential neuroprotective compounds (Miguel-Hidalgo et al. 2002, Sun et al. 2006, Helton et al. 2008). Alone, exposure to glutamate diminished cellular viability in CGNs by almost 50%, as assessed by MTT assay. However, co-treatment of the cells with either 0.15% SAE or 0.25% BAE abrogated this effect, preserving cell viability at a level that was comparable to untreated control cells (Fig. 2.3A).

While excitotoxicity is known to contribute to neurodegenerative disease processes, and particularly to oxidative stress, other factors, such as neuroinflammation, also play major roles in promoting cell death through production of free radical species. Indeed, a key aspect of neuroinflammation is the release of toxic nitric oxide radicals by astrocytes and microglia, which are capable of promoting conditions of nitrosative stress in neighboring neurons that ultimately culminate in the activation of cell death signaling cascades (Di Filippo et al. 2010). Since these processes do not occur independently of other disease factors such as excitotoxicity, it is important that potential therapeutics for neurodegeneration are capable of mitigating both oxidative and nitrosative stress in order

to target multiple facets of disease pathology. To this end, we next tested the ability of the two anthocyanin extracts to defend cells from sodium nitroprusside (SNP), a nitric oxide donor, meant in this context to simulate conditions of nitrosative stress. Cells treated with SNP alone showed a drastic reduction in viability of 95%, and treatment with 0.15% SAE demonstrated no ability to preserve cellular viability in the presence of this insult (Fig. 2.3B). In striking contrast, treatment with 0.25% BAE significantly preserved neuronal survival in the presence of SNP (Fig. 2.3B).

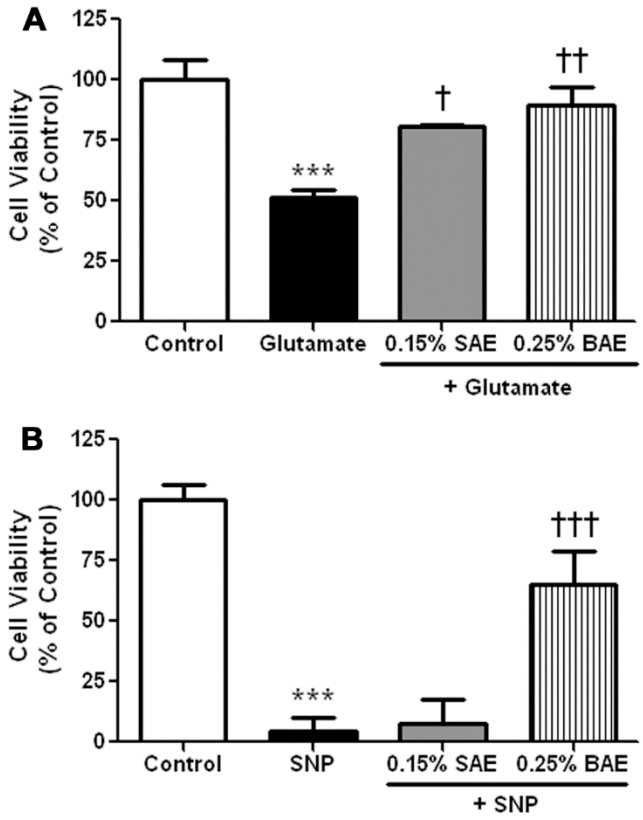

Figure 2.3. Effects of SAE and BAE on glutamate excitotoxicity and nitric oxide toxicity induced by sodium nitroprusside (SNP). *A,* Primary rat cerebellar granule neurons (CGNs) were treated with 100µM glutamate alone or in combination with 0.15% SAE or 0.25% BAE for 24h. Cell viability was then assessed by MTT assay; *B,* CGNs were treated with 100µM SNP alone or in combination with SAE or BAE for 24h. Cell viability was then assessed by MTT assay. All data are represented as mean ± SEM. *** indicates $p<0.001$ compared to untreated control cells, ††† represents $p<0.001$, †† represents $p<0.01$, and † represents $p<0.05$ in comparison to cells treated with the insult alone as determined by one-way ANOVA with a post hoc Tukey's test.

2.4.2 Both Callistephin and Kuromanin, the Primary Anthocyanin Constituents of SAE and BAE, Protect CGNs from Glutamate Excitotoxicity

The differential protective effects of the two anthocyanin extracts were thought to be attributed to differences in the neuroprotective potential of their respective primary anthocyanin constituents. However, given that neither extract was composed purely of a single anthocyanin, the possibility that the differential neuroprotective abilities were due to other compounds contained in the extract, or synergism between several compounds, could not be excluded. Therefore, in order to confirm that the observed differences in neuroprotection corresponded to the differing properties of the two primary anthocyanins, callistephin and kuromanin, we next examined the neuroprotective capabilities of the two pure anthocyanins.

Pure preparations of both callistephin and kuromanin were obtained commercially and tested for their ability to protect CGNs from glutamate excitotoxicity. Because pure anthocyanins were used at a higher concentration than either of the extracts and produced deeply pigmented solutions with absorbance in the same range as MTT products, cells were fixed following treatment, and nuclear morphology was observed in order to quantify apoptosis. In general, cells displaying condensed and/or fragmented nuclei were scored as apoptotic. In a preliminary dose response, it was found that pure anthocyanins were less efficient neuroprotective agents than the extracts, requiring a dose of either 75μM or 100μM for kuromanin and callistephin respectively in order to significantly protect CGNs from excitotoxic insult (Winter and Linseman, unpublished data). Given this observation, a dose of 100μM was selected to complete all subsequent analyses.

Treatment with glutamate alone produced death in approximately 50% of CGNs, consistent with our previous observations using the MTT assay (compare Fig. 2.3A and 2.4B). These cultures displayed a much higher proportion of cells with condensed or fragmented nuclei in addition to a compromised tubulin network when compared to untreated controls (Fig. 2.4A). This result is consistent with induction of neuronal apoptosis. Intriguingly, treatment with either callistephin or kuromanin significantly reduced the number of cells displaying apoptotic nuclei and fragmented tubulin (Fig. 2.4A). Quantitatively, callistephin and kuromanin reduced CGN apoptosis to 20% and 15% respectively, which is in good agreement with our previous observations using SAE and BAE against glutamate excitotoxicity (compare Fig. 2.3A and 2.4B). Collectively, these results suggest that callistephin and kuromanin, the primary anthocyanin constituents of SAE and BAE, respectively, are capable of mediating similar neuroprotective effects against glutamate-induced excitotoxicity to those observed using enriched anthocyanin extracts.

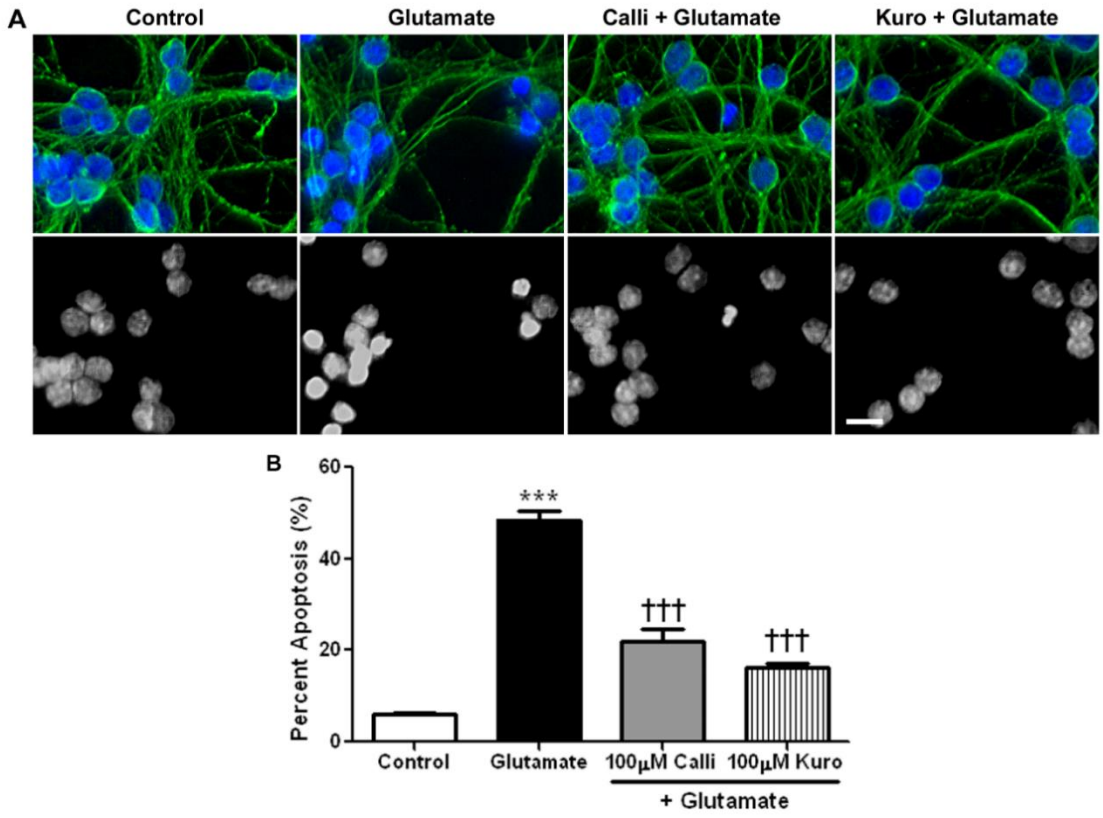

Figure 2.4. Both pure callistephin and kuromanin protect CGNs from glutamate-induced excitotoxicity. *A,* Representative fluorescent micrographs showing untreated control CGNs, or CGNs treated with 100μM glutamate alone or in combination with 100μM callistephin (Calli) or 100μM kuromanin (Kuro) for 24 hours. Top panels show immunocytochemistry for β-tubulin (green) and Hoechst stain (blue). Panels on the bottom show decolorized Hoechst fluorescence to visualize nuclear morphology. Scale bar=10μm; *B,* Quantitative assessment of cellular apoptosis CGNs treated as described in *(A)*. Cells were counted and scored as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are represented as the mean ± SEM. *** indicates $p<0.001$ compared to untreated control cells and ††† represents $p<0.001$ in comparison to cells treated with glutamate alone, as determined by one-way ANOVA with a *post hoc* Tukey's test.

2.4.3 Kuromanin, But Not Callistephin, Protects CGNs from Nitric Oxide-Induced Toxicity

In order to further compare the neuroprotective capacity of pure anthocyanin compounds to neuroprotection mediated by SAE and BAE, callistephin and kuromanin were next assessed for their ability to defend CGNs from nitric oxide-induced toxicity. CGNs that were treated with SNP alone uniformly displayed condensed and fragmented nuclear morphology as well as extensive disintegration of tubulin networks in comparison to untreated controls (Fig. 2.5A). Quantification of condensed nuclei revealed that 95% of cells exposed to SNP alone underwent apoptosis in concurrence with our previous results using MTT (compare Fig. 2.3B and 2.5B). Interestingly, cells that were co-treated with both callistephin and SNP displayed the same features as cells treated with SNP alone, and no significant reduction in the number of apoptotic nuclei was observed (Fig. 2.5A, B). In stark contrast to this observation, co-treatment with kuromanin dramatically mitigated these effects, preserving tubulin networks and maintaining healthy nuclear morphology (Fig. 2.5A). These cells showed a reduction in apoptosis of nearly 70% in comparison to cells treated with SNP alone, or SNP with callistephin, consistent with the protective effect if BAE against SNP demonstrated using MTT (compare Fig. 2.3B and 2.5B).

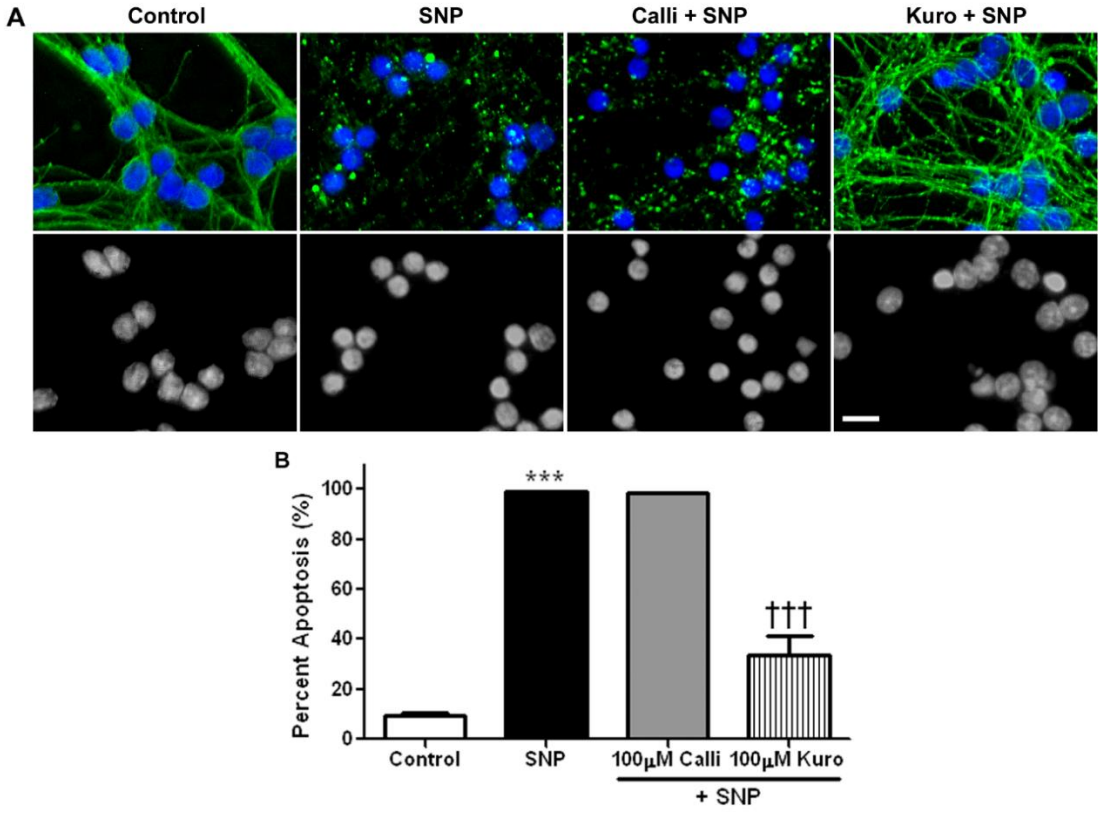

Figure 2.5. Pure kuromanin, but not callistephin, protects CGNs from nitric oxide-induced toxicity. *A,* Representative fluorescent micrographs showing untreated control CGNs, or CGNs treated with 100μM SNP alone or in combination with 100μM callistephin (Calli) or 100μM kuromanin (Kuro) for 24 hours. Top panels show immunocytochemistry for β-tubulin (green) and Hoechst stain (blue). Panels on the bottom show decolorized Hoechst fluorescence to visualize nuclear morphology. Scale bar=10μm; *B,* Quantitative assessment of cellular apoptosis in CGNs treated as described in *(A)*. Cells were counted and scored as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are represented as the mean ± SEM. *** indicates $p<0.001$ compared to untreated control cells and ††† represents $p<0.001$ in comparison to cells treated with SNP alone, as determined by one-way ANOVA with a *post hoc* Tukey's test.

2.4.4 Kuromanin, But Not Callistephin, Reduces Nitric Oxide Concentration in a Cell Free System and Produces Superoxide

Given the stark contrast between the abilities of callistephin and kuromanin to prevent the harmful effects of nitrosative stress in neurons, a nitric oxide assay was used to determine the final concentration of nitric oxide in cell-free medium incubated with callistephin or kuromanin and SNP. Analysis using a Griess assay to measure total nitrite and nitrate, decomposition products of nitric oxide, demonstrated that culture medium incubated overnight with SNP produced a substantial amount of nitric oxide in solution (Fig. 2.6A). Consistent with its inability to defend CGNs from nitric oxide toxicity, co-incubation with callistephin did not reduce the concentration of nitric oxide in solution (Fig. 2.6A). Incubation with kuromanin, however, significantly decreased the amount of nitric oxide that remained in solution following generation by SNP, suggesting that this anthocyanin is able to scavenge nitric oxide in some capacity (Fig. 2.6A).

We next aimed to determine the chemical mechanism by which kuromanin is able to scavenge nitric oxide, and prevent subsequent toxicity, in contrast to callistephin. These anthocyanins share a significant degree of structural homology at the molecular level, differing only by the presence of a single hydroxyl group on the B-ring of kuromanin (Fig. 2.1). Given that these two compounds are otherwise identical, we postulated that the presence or absence of this catechol moiety on the B-ring is responsible for the differential neuroprotection of the anthocyanins against nitric oxide-induced cell death.

One unique aspect of catechol chemistry is the ability of catecholic compounds, such as kuromanin, to auto-oxidize and generate superoxide (Chichirau et al. 2005). The superoxide produced from these reactions is highly reactive and combines readily with other radical species such as nitric oxide. Therefore, we next assessed the ability of kuromanin to generate superoxide in a cell free system.

2-Methyl-6-(4-Methoxyphenyl) -3,7-Dihydroimidazo[1, 2-A]pyrazin-O-3ne, Hydrochloride (MCLA) luminescence was used to detect the presence of superoxide in solutions containing 100μM kuromanin alone or in combination with quinone oxidoreductase 1 (QR1) or quinone oxidoreductase 2 (QR2) in a cell free system. At this concentration, kuromanin alone did not produce detectable concentrations of superoxide in solution (Fig. 2.6B). Given that this assay was performed in the absence of cells and associated reducing factors that would allow kuromanin to undergo multiple auto-oxidation reactions, this result was not altogether unexpected. Without these reducing factors, each molecule has the potential to undergo only one auto-oxidation reaction, and detection would be limited by the rate at which this reaction occurred. QR1 and QR2 were added to the reaction to act as generic reducing factors for the quinone formed when kuromanin is fully oxidized, creating the potential for each molecule of kuromanin to undergo multiple oxidation reactions. Addition of QR1 to the system produced a modest luminescence signal while addition of QR2 significantly enhanced MCLA luminescence (Fig. 2.6B). Neither QR1 nor QR2 produced an appreciable luminescence signal when incubated alone (Winter and Linseman, unpublished data). Addition of SOD1 to solutions containing kuromanin and QR2 completely quenched MCLA luminescence, confirming

that superoxide, and not another reactive radical species, was responsible for the observed increase in the luminescence signal (Fig. 2.6B).

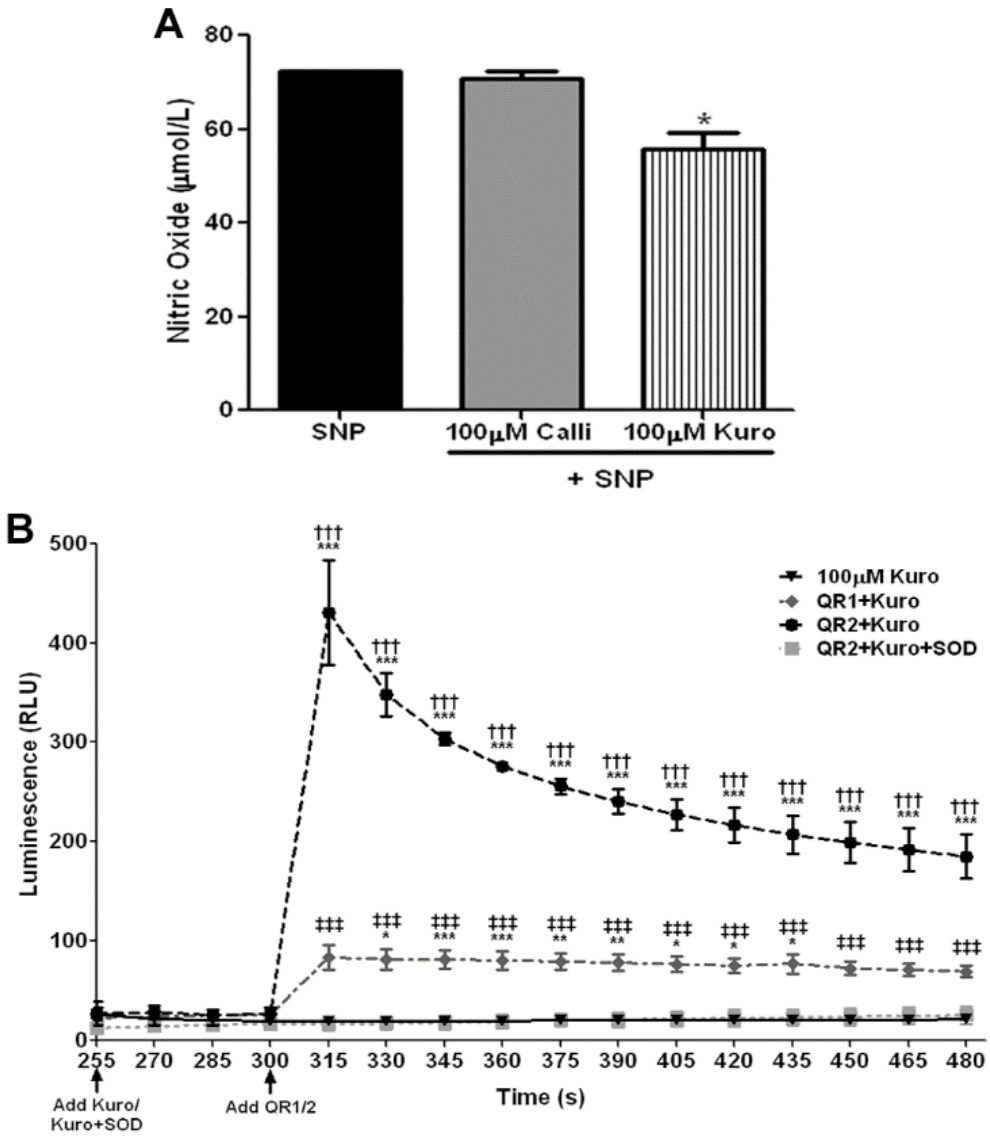

Figure 2.6. Kuromanin, but not callistephin scavenges nitric oxide present in culture medium following incubation with SNP through a superoxide dependent mechanism. *A,* 100µM SNP was incubated in fresh, serum free, culture medium at 37°C alone or in combination with 100µM callistephin (Calli) or 100µM kuromanin (Kuro) for 24 hours to mimic cell culture conditions. The amount of total nitrate and nitrite was then assessed in culture medium using the Griess assay to quantify the amount of nitric oxide remaining after anthocyanin addition. Each experiment was run in parallel with a sample containing only culture medium and no SNP. The culture medium did not contain any nitrates or nitrites that would be detected by the assay, but was calculated to have an average nitrate/nitrite concentration of approximately 15.704µM due to absorbance by phenol red in the culture medium. This value was therefore subtracted from the total concentration of nitric oxide detected by the Griess Assay for the three remaining

samples. These adjusted values are reported here. Data are represented as the mean ± SEM. * indicates $p<0.05$ compared to culture medium containing SNP alone by one-way ANOVA with post hoc Tukey's test; *B,* Superoxide production by kuromanin was monitored using MCLA luminescence assay. MCLA luminescence was recorded at intervals of 15 seconds for a total of 8 minutes. A baseline was recorded for the first 4 minutes of the assay (data not shown) before addition of 100μM kuromanin alone or in combination with 30U/mL Cu, Zn-superoxide dismutase (SOD1). MCLA luminescence was then assessed for 1 minute before the addition of 5U/mL of either quinone oxidoreductase 1 or 2 (QR1/2) and their appropriate co-factors. Time points at which additions were completed are indicated below the x-axis of the graph. *** indicates $p<0.001$, ** indicates $p<0.01$, and * indicates $p<0.05$ compared to MCLA luminescence with kuromanin alone, ††† indicates $p<0.001$ compared to MCLA luminescence in sample containing kuromanin, QR2, and SOD1, and ‡‡‡ indicates $p<0.001$ compared to samples containing kuromanin and QR2. All statistics were conducted using two-way ANOVA for repeated measures with a post hoc Bonferroni test to determine differences between groups.

2.4.5 Kuromanin Protects Neurons from Nitric Oxide Toxicity in a Superoxide-Dependent Manner

To validate the data obtained using a cell free approach, we next examined the role of superoxide production in defending cultured neurons from nitric oxide toxicity. As before, CGNs were cultured with SNP alone, or in the presence of kuromanin, and evaluated for apoptosis. In addition to these treatment groups, cells were also treated with kuromanin and SNP in the presence of PEG-SOD, a cell permeable conjugate of SOD1, to scavenge any superoxide generated in culture. In agreement with our previous results, cells treated with SNP alone displayed fragmented tubulin networks and condensed nuclei indicative of widespread apoptosis, in comparison to untreated control cells (Fig. 2.7A). Kuromanin effectively mitigated these effects, preserving tubulin networks and healthy nuclear morphology. However, in the presence of PEG-SOD, the neuroprotective effects of kuromanin were completely ablated. Cells treated with PEG-SOD showed extensive disintegration of tubulin networks and condensed nuclei similar to cells that were treated with SNP alone (Fig. 2.7A). This effect was concentration dependent, as low concentrations of PEG-SOD had essentially no influence on the protective abilities of kuromanin, while concentrations of 30U/mL or more completely abolished kuromanin-mediated neuroprotection (Fig. 2.7B). At these concentrations, up to 97% of cells possessed condensed or fragmented nuclei, and no significant difference was observed between the percentage of apoptotic cells under these conditions in comparison to cells treated with SNP alone (Fig. 2.7B). Moreover, treatment with PEG-SOD alone at the

concentrations tested in these experiments had no effect on cellular viability (Winter and Linseman, unpublished data).

Following these experiments, we next sought to determine if superoxide generation by kuromanin occurred at the extracellular or intracellular level by comparing the effects of PEG-SOD and unconjugated SOD1, which is not cell permeable. As before, kuromanin protected CGNs from nitric oxide-induced toxicity, and these effects were blocked by concomitant treatment with PEG-SOD. However, treatment with unconjugated SOD1 had no significant effect on the neuroprotective capacity of kuromanin, suggesting that these effects occur at the intracellular level (Fig. 2.7C).

To confirm this observation using a molecular approach, we transfected Neuro2A mouse neuroblastoma cells with either a control vector expressing EGFP or a vector expressing EGFP-tagged, wild type SOD1, and treated them with SNP alone or in the presence of kuromanin. We then assessed the number of transfected cells displaying condensed or fragmented nuclei indicative of cell death. Cells transfected with the EGFP vector and treated with SNP alone underwent significant cell death in comparison to untreated EGFP-transfected controls, while cells that were co-treated with both SNP and kuromanin displayed a significant reduction in cell death in comparison to cells treated with SNP alone (Fig. 2.7D). In contrast, cells transfected with SOD1 and treated with SNP and kuromanin displayed the opposite result. Indeed, SOD1 overexpressing cells showed a marked increase in cell death in comparison to EGFP-transfected cells that were treated with SNP and kuromanin, and this level of death was comparable to that observed when cells were treated with SNP alone (Fig. 2.7D).

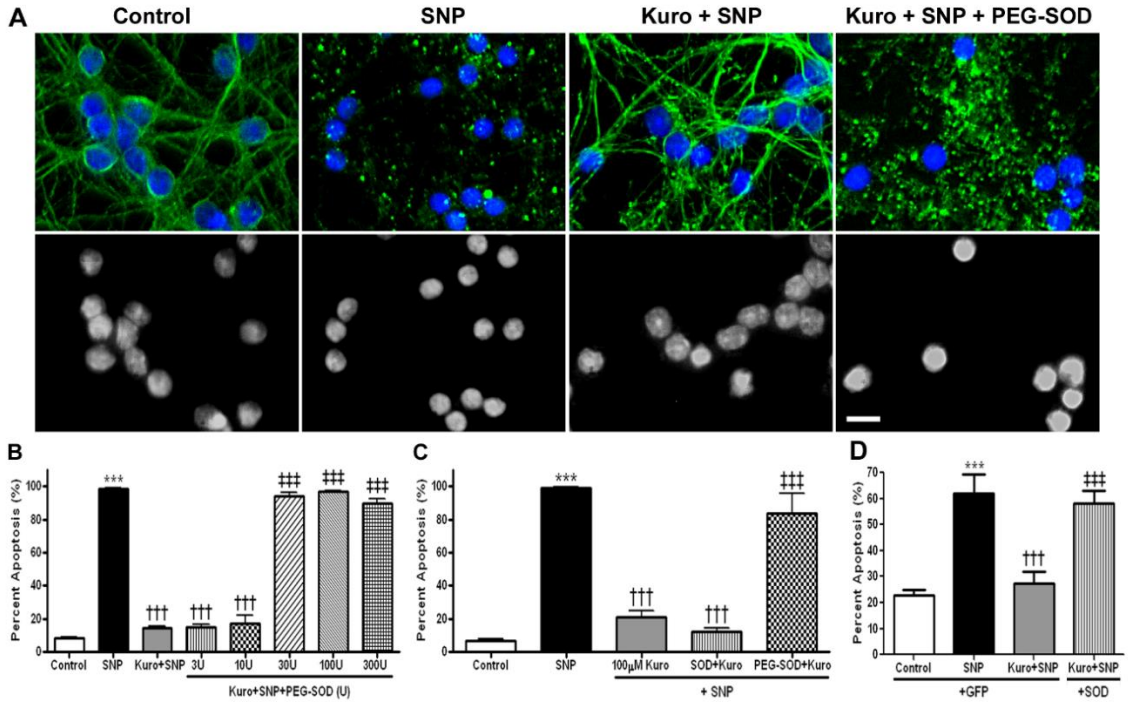

Figure 2.7. Delivery of SOD1 intracellularly blocks the neuroprotective effects of kuromanin against SNP. *A,* Representative fluorescent micrographs showing untreated control CGNs, CGNs treated with 100μM SNP alone or in combination with 100μM kuromanin (Kuro), and CGNs treated with SNP, kuromanin, and PEG-SOD at the indicated concentrations (U/mL) for 24h. Top panels show immunocytochemistry for β-tubulin (green) and Hoechst stain (blue). Panels on the bottom show decolorized Hoechst fluorescence to visualize nuclear morphology. Scale bar=10μm; *B,* Quantitative assessment of cellular apoptosis for CGNs treated as in *(A)*. Cells were counted and scored as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined; *C,* Quantitative assessment of cellular apoptosis for untreated control CGNs, CGNs treated with SNP alone or in combination with kuromanin, and CGNs treated with SNP, kuromanin, and either unconjugated SOD1 or PEG-SOD for 24 hours; *D,* Quantitative assessment of apoptosis in Neuro2A cells transfected with either EGFP or EGFP-tagged SOD1 and treated with 200μM SNP alone or in combination with 100μM kuromanin. Transfected (EGFP-positive) cells were counted and scored as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are represented as the mean ± SEM. *** indicates $p<0.001$ compared to untreated control cells, ††† represents $p<0.001$ in comparison to cells treated with SNP alone, and ‡‡‡ indicates $p<0.001$ in comparison to cells treated with kuromanin and SNP in combination as determined by one-way ANOVA with a *post hoc* Tukey's test.

2.5 Discussion

Initially, two anthocyanin-enriched extracts were prepared from freeze-dried strawberry or blackberry powder in order to preliminarily characterize the neuroprotective effects of two distinct anthocyanin compositions. These fruits were chosen as their primary anthocyanin constituents, callistephin and kuromanin, have been previously described and are known to compose a majority of the anthocyanin profiles of strawberries and blackberries, respectively (Elisia et al. 2007, Baiamonte et al. 2010). This increased the likelihood that any observed protective effects would be due to these two anthocyanins and not another phenolic species within the extracts. While it was found that treatment with either SAE or BAE was sufficient to defend primary neurons from excitotoxic insult, a stark contrast in neuroprotective ability between these two extracts was observed in cells treated with the nitric oxide donor, SNP. While BAE was able to significantly protect neurons from nitric oxide toxicity, SAE had no observable effect against this insult, suggesting that there is a distinct difference between the neuroprotective effects of the primary anthocyanin constituents of the two extracts. Similar effects were observed when pure preparations of both callistephin and kuromanin were assessed for their neuroprotective potential, confirming that these two compounds were likely responsible for the results observed when using enriched fruit extracts. However, it was notable that the protective concentration of kuromanin and callistephin in the extracts was much lower than that observed for the pure compounds. This may suggest synergism with other anthocyanin species or contaminating phenolics within the extract which was not observed when treating with the individual anthocyanins, and

illustrates one of the confounding factors of assessing the neuroprotective potential of particular species within enriched extracts. Taken as a whole, these results indicate that subtle molecular differences between callistephin and kuromanin, specifically the absence or presence of a catechol moiety on the B-ring, was responsible for the differential neuroprotective effects observed under conditions of nitric oxide toxicity. This hypothesis was supported by the observation that kuromanin, which contains a catechol as part of its B-ring, was able to scavenge nitric oxide in solution to a significant degree, while callistephin, which lacks a catechol, was not.

Interestingly, the chemistry of anthocyanins and many other natural products is such that these potent antioxidants can behave as pro-oxidants under certain conditions, such as at high concentrations (Yen et al. 2003, Prochazkova et al. 2011). In particular, catechol containing compounds such as kuromanin are thought to execute their pro-oxidant functions through the process of auto-oxidation. This reaction proceeds using molecular oxygen as an electron acceptor to produce first a semiquinone and then a quinone molecule with a net production of one superoxide radical (Fig. 2.8; Chichirau et al. 2005). Within the cell where there are multiple reducing factors that may regenerate the original parent compound, this auto-oxidation cycle could proceed many times for a single molecule, increasing the abundance of superoxide that is ultimately produced.

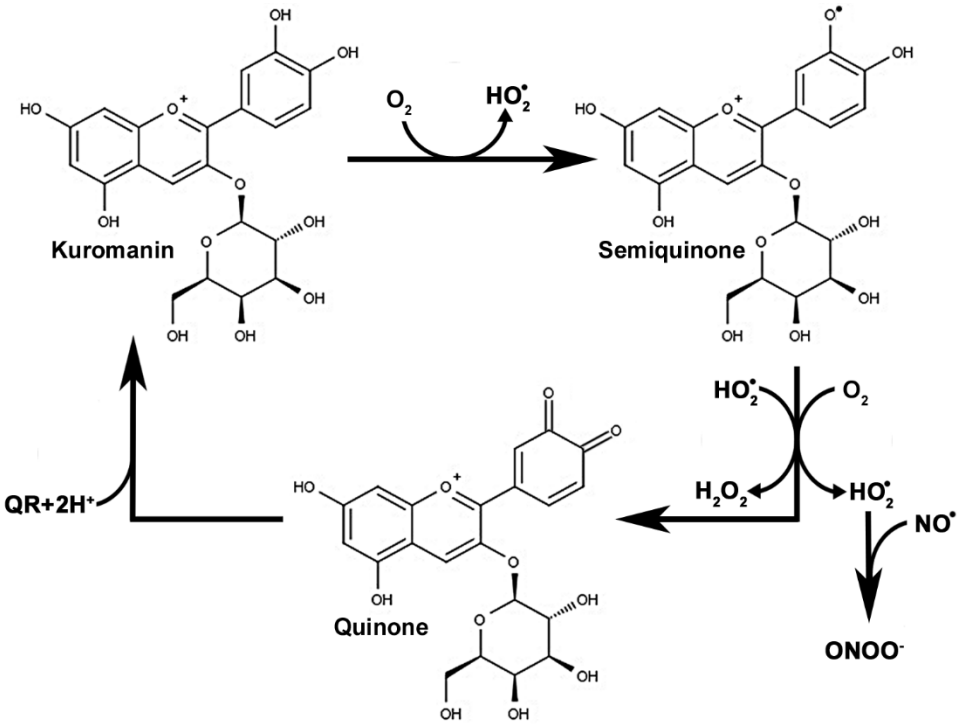

Figure 2.8. Proposed auto-oxidation reaction of kuromanin to produce superoxide. Kuromanin reacts with molecular oxygen to produce a semiquinone and one superoxide radical (HO2•). Superoxide produced from this reaction is consumed to produce hydrogen peroxide (H_2O_2), while molecular oxygen interacts with the semiquinone radical to form more superoxide. This results in the net production of one molecule of H_2O_2, one superoxide radical, and the oxidized quinone form of kuromanin. The superoxide radical produced from this reaction is then able to scavenge nitric oxide (NO•) radicals to form peroxynitrite ($ONOO^-$). The oxidized quinone form of kuromanin is then recycled to the reduced catechol by a quinone reductase or other reducing factor (QR). This mechanism is adapted from a mechanism proposed by Chichirau et al. (37).

This hypothesis is consistent with our observation that kuromanin produces superoxide, but this radical reached detectable concentrations only when kuromanin was incubated in the presence of a quinone reductase. Moreover, Chichirau et al. (2005) propose that this reaction may only occur while the parent catechol is in its anionic form, which accounts for only a small proportion of aqueous anthocyanins at a given time, further hampering detection of superoxide. While both quinone reductases used in our experiments caused some increased superoxide production by kuromanin, the largest increase in superoxide production was observed in the presence of QR2. These results are consistent with the observation that QR1 works best on *meta*- and *para*-quinones, with a low affinity for *ortho*-quinones, while QR2 preferentially reduces *ortho*-quinones, as would be formed by kuromanin (Fu et al. 2008). This also lends further support to the idea that catechol-containing species like kuromanin are not only capable of generating superoxide, but may do so with an enhanced capacity in the presence of appropriate reducing factors, as might occur within a cell.

Pro-oxidant processes are thought to be partially responsible for the anti-carcinogenic properties of anthocyanins and other nutraceuticals, and have spurred the development of the "quercetin paradox." This paradox suggests that by performing their antioxidant function, flavonoids and other polyphenols produce reactive species that are capable of depleting endogenous antioxidants like glutathione, ultimately causing cytotoxicity (Weisburg et al. 2004, Lee et al. 2005, Boots et al. 200, Feng et al. 2007). It is noteworthy, then, that both callistephin and kuromanin were previously demonstrated to preserve neuronal viability and glutathione levels in the presence of the glutathione-

depleting agent, HA14-1, which may suggest that anthocyanin chemistry is uniquely suited to avoid glutathione depletion, unlike quercetin (Boots et al. 2007, Kelsey et al. 2011). This is further supported by the observation that an anthocyanin-rich diet stimulates promoter activity for γ-glutamylcysteine ligase, the rate limiting enzyme in glutathione synthesis, and increases glutathione levels in kidney, liver, and brain tissue *in vivo* (Carlsen et al. 2003, Hassimotto et al. 2011). Most impressive is the observation that in the context of nitric oxide toxicity and subsequent nitrosative stress, superoxide generated through auto-oxidation of kuromanin appears to play the unexpected role of a neuroprotective nitric oxide scavenger rather than that of a toxic free radical, illustrating a role for the pro-oxidant activity of anthocyanins in mitigating this facet of neurodegenerative disease. Indeed, incorporation of excess SOD1 into the cell culture completely abolished the neuroprotective effect of kuromanin against nitric oxide, indicating that this process is superoxide-dependent. Moreover, this phenomenon was observed only when SOD1 was able to enter the cell, either as a cell-permeable form of the enzyme, PEG-SOD, or through transfection with wild type SOD1, demonstrating that nitric oxide scavenging by superoxide takes place at the intracellular level. This is significant in that damage from nitric oxide occurs largely at the intracellular level throughout many neurodegenerative processes, either by induction of nitric oxide synthase (NOS) through inflammatory cytokine signaling, or through diffusion into neuronal cells from neighboring glia (Raoul et al. 2006, Uehara et al. 2006).

Combination of superoxide and nitric oxide results in the formation of peroxynitrite. While high levels of peroxynitrite can also be toxic, this compound has a

very short half-life and is readily scavenged by a number of endogenous antioxidants, such as glutathione, in addition to flavonoids such as kuromanin and quercetin, that possess a catechol as a part of their B-ring (Heijnen et al. 2001). Strikingly, there is also evidence to suggest that, in moderation, peroxynitrite is neuroprotective against nitric oxide toxicity in primary cortical neurons (Garcia-Nogales et al. 2003, Delgado-Esteban et al. 2007). These effects are due to stimulation of the pentose phosphate pathway, which generates an abundance of NADPH that can be used in the synthesis of important antioxidants such as glutathione, and other cellular defenses (Garcia-Nogales et al. 2003). Additionally, oxidative modification of phosphatase tensin homolog (PTEN) by peroxynitrite has been shown to elicit a neuroprotective response by enhancing activation of the PI3K/Akt pro-survival pathway (Delgado-Esteban et al. 2007). Taken together, these data lead us to conclude that peroxynitrite generated through the proposed scavenging mechanism illustrated in Figure 8 is not only non-toxic, but may in fact contribute to the neuroprotective effect of kuromanin against nitric oxide.

Our structure-activity analysis of callistephin and kuromanin indicates that although all anthocyanins bear a high degree of structural homology, the absence or presence of a catechol moiety on the B-ring of the compound is necessary for it to mitigate nitric oxide toxicity and subsequent cell death. This may also be true of delphinidin-based anthocyanins, and compounds such as epigallocatechin gallate, which possess a pyrogallol moiety as part of their structure. Such compounds are also capable of producing superoxide and reactive *ortho*-quinones through the process of auto-oxidation, similar to kuromanin (Li, 2012). However, it should be noted that while these compounds

may undergo similar processes, and therefore protect similarly from nitric oxide toxicity, various anthocyanins also display differences in their abilities to interact with other oxidative species. For example, increasing hydrophilicity in anthocyanins through addition of hydroxyl groups lowers the ability of these compounds to interact with lipid-rich environments, hampering their ability to prevent other prominent features of neurodegeneration such as lipid peroxidation (Brown and Kelly, 2007). It is also notable that while most common anthocyanins are known to cross the blood-brain barrier (BBB), highly hydrophilic anthocyanins interact less with lipid-rich environments, and cross the BBB at a lower rate than their more lipophilic flavonoid relatives in an *in vitro* model of the BBB (Youdim et al. 2003, Brown and Kelly, 2007, Faria et al. 2014) This decreases the likelihood that anthocyanins that contain a high number of hydroxyl groups, particularly on the B-ring, can effectively mitigate oxidative damage in the brain. While further evidence is required to confirm this observation *in vivo*, it has been previously observed that the rate of transport across the basolateral membrane during digestion is directly correlated with the lipophilicity of the anthocyanin in question (Yi et al. 2006). As a result, compounds such as delphinidin-based anthocyanins may prove to be less effective therapeutics than other more lipophilic anthocyanins like kuromanin.

This highlights the importance of choosing a drug candidate with versatile chemistry to target multiple aspects of neurodegenerative disease in the most effective way possible. For this reason, mechanistic studies examining individual compounds for their efficacy in various systems that mimic neurodegenerative conditions are advantageous. Moreover this approach also lends itself to the creation of a multi-agent

treatment paradigm, which may ultimately be required to combat complex neurodegenerative diseases, as highly effective compounds with complimentary chemical properties can be readily identified and combined in high through-put screens to assess potential synergism. This allows for the rapid assessment and development of new drug combinations for further preclinical testing.

While it is true that some element of the multi-agent strategy is present in the use of enriched fruit extracts, it is often the case that a single extract may contain compounds that directly hinder one another's performance, compromising its effectiveness. Additionally, such extracts are difficult to standardize and purify sufficiently to ensure safety during consumption for clinical development. Lastly, understanding the mechanistic aspects of neuroprotection for a single compound, such as kuromanin, may allow for the identification or design of similar substances that are able to perform the same functions with even greater efficiency, which cannot be accomplished using extracts. In the case of anthocyanins, this may be especially true of their phenolic acid metabolites, which are derived from the B-ring of the parent compounds, and appear at concentrations in the body that far exceed that of the parent anthocyanin following ingestion (Tsuda et al. 1999, Azzini et al. 2010). Thus, it will be of great interest in the future to determine if anthocyanin metabolites behave in a manner similar to their parent compounds, as these derivatives may ultimately be responsible for the observed benefits of anthocyanin consumption *in vivo* due to their higher bioavailability.

Collectively, our data demonstrate that some anthocyanins may be more uniquely suited than others to combat the multi-faceted nature of neurodegenerative disorders.

Indeed, while anthocyanins appear to be universally capable of mitigating conditions of oxidative stress to some degree, our observations clearly show that this ability does not extend to all anthocyanin family members under conditions of nitrosative stress. This understanding is vital for determining which anthocyanin species have the highest potential as preventative and therapeutic agents in the context of neurodegeneration, and for the development of new, more effective therapeutics in the future.

CHAPTER THREE: AN ANTHOCYANIN-ENRICHED EXTRACT FROM STRAWBERRIES DELAYS DISEASE ONSET AND EXTENDS SURVIVAL IN THE HSOD1^{G93A} MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS.

3.1 Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease that results from the death of motor neurons in the brain, brain stem, and spinal cord. While the etiology of ALS is poorly defined, recent data indicate that there are many pathological contributors to disease progression such as oxidative stress, neuroinflammation, and neuronal apoptosis. Anthocyanins are flavonoid compounds derived from many common fruits and vegetables that possess pleiotropic effects, acting as antioxidant, anti-inflammatory, and anti-apoptotic agents. Thus, these unique compounds may provide therapeutic benefit for the treatment of ALS. Here, we used the G93A mutant human SOD1 (hSOD1^{G93A}) mouse model of familial ALS to assess the effects of an anthocyanin-enriched extract from strawberries (SAE) on disease onset and progression. Transgenic hSOD1^{G93A} mice that received SAE supplementation presymptomatically experienced a marked (~17 day) delay in disease onset and a statistically significant (~11 day) extension in survival in comparison to untreated hSOD1^{G93A} mice. Additionally, hSOD1^{G93A} mice treated with SAE displayed a

significant preservation in paw grip strength throughout disease progression in comparison to their untreated mutant counterparts. Histopathological analysis showed that SAE supplementation significantly reduced astrogliosis in spinal cord, and preserved neuromuscular junctions in gastrocnemius muscle. These data are the first to demonstrate that anthocyanins have significant impact as therapeutic agents in a preclinical model of ALS. Therefore, further study of these compounds is warranted in additional preclinical models and patients suffering from ALS and possibly other neurodegenerative diseases.

3.2 Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the death of motor neurons in the motor cortex, brainstem and spinal cord. Recent evidence suggests that oxidative stress and inflammation of glial cells within the CNS are major contributing factors to the apoptotic death of motor neurons in ALS. Markers of oxidative stress are observed in tissue collected from both sporadic and familial ALS patients. These include reduced levels of the critical antioxidant, glutathione, in blood and brain, and elevations in oxidative damage to DNA and lipids (Bogdanov et al. 2000, Simpson et al. 2004, Babu et al. 2008, Weiduschat et al. 2014). Similarly, indices of neuroinflammation are also found in abundance in tissue from both ALS patients and mouse models of the disease. Microglia and astrocytes have been shown to play significant roles in modulating ALS disease progression in mutant SOD1 mouse models (Beers et al. 2006, Boilée et al. 2006, Yamanaka et al. 2008, Wang et al. 2011). These findings are supported by data collected from spinal cord tissue and

cerebrospinal fluid from ALS mouse models and patients which display significant elevations in a number of pro-inflammatory cytokines and enzymes, such as tumor necrosis factor-α, cyclooxygenase-2 and inducible nitric oxide synthase, products of inflamed glial cells known to cause motor neuron death (Almer et al. 1999, Almer et al. 2001, Hensley et al. 2003, Raoul et al. 2006).

Both oxidative stress and neuroinflammation are thought to be significant contributors to the induction of apoptosis in affected motor neurons, highlighting the complex nature of this disease. However, the exploration of new treatments for ALS thus far has largely been targeted at mitigating only a single aspect of ALS pathology, a strategy that has proven unsuccessful up to this point. For example, the only drug approved to treat ALS, Riluzole, is aimed specifically at attenuating the damaging effects of excitotoxicity; however, its effect on disease course is modest, extending lifespan only 2-3 months (Miller et al. 2012). As such, focus on treating ALS has recently begun to shift towards the use of agents that, via their molecular mechanism of action, target multiple aspects of the underlying disease pathology.

Anthocyanins are a unique class of flavonoid compounds responsible for the red, blue, and purple coloration of many fruits, vegetables and flowers. These compounds have been shown to possess a considerable antioxidant capacity *in vitro* and have been demonstrated to display neuroprotective effects against oxidative insults such as hydrogen peroxide (Ghosh et al. 2006, Heo and Lee 2005, Tarozzi et al. 2007, Zafra-Stone et al. 2007, Spada et al. 2009, Zhu et al. 2010). Furthermore, anthocyanins have recently garnered attention as anti-inflammatory agents, significantly reducing

inflammatory responses induced by lipopolysaccharide stimulation in BV2 microglial cells (Lau et al. 2007, Poulose et al. 2012, Carey et al. 2013, Jeong et al. 2013). Anthocyanins have also been shown to directly modulate pro-survival and pro-apoptotic signaling pathways, further contributing to their neuroprotective effects (Shin et al. 2006, Reddivari et al. 2007, Lu et al. 2010, Kim et al. 2010, Ye et al. 2010, Min et al. 2011). The pleiotropic nature of these compounds makes them highly appealing as potential therapeutics for the treatment of diseases like ALS that possess a complex etiology and progression characterized by both oxidative stress and inflammation. We have previously shown that an anthocyanin-enriched extract from strawberries (SAE) and its primary anthocyanin constituent, callistephin, display significant neuroprotective effects against glutamate excitotoxicity and mitochondrial oxidative stress *in vitro* (Kelsey et al. 2011, Chapter 2, Section 2.4). Therefore, we sought to explore the therapeutic potential of SAE in the G93A mutant human Cu, Zn-superoxide dismutase ($hSOD1^{G93A}$) mouse model of familial ALS.

3.3. Materials and Methods

3.3.1 Anthocyanin Extraction and HPLC Analysis

Anthocyanins were extracted from commercially available Nutri-Fruit freeze-dried strawberry powder (Scenic Fruit Co., Gresham Oregon) using the protocol established by Rodriguez-Saona and Wrolstad (2001). Briefly, 500g of freeze-dried powder was macerated in a large flask with acidified methanol (0.01% HCl) to obtain a crude extract of all (poly)phenolic species. The extract was collected by filtration using

Whatman no. 1 filter paper, and the remaining powder was re-extracted with acidified methanol until a faintly colored solution was obtained. The crude extract was pooled together, then boiled under vacuum to remove the methanol until a viscous solution was obtained. This solution was then purified for anthocyanins using a C18 column prepared with acidified water (0.01% HCl) by loading one column volume of the crude extract onto the column followed by sequential washes with one column volume each of acidified water and ethyl acetate. Anthocyanins were then eluted into a separate collection flask by passing one column volume of acidified methanol (0.01% HCl) through the column. This extract, containing purified anthocyanins, was then boiled under vacuum until a strongly pigmented and viscous solution was obtained.

The purified strawberry anthocyanin extract (SAE) was analyzed and the primary anthocyanin constituent was identified using HPLC. All analyses were conducted using HPLC-UV/Vis with an Agilent model HP1100 series II with programmable diode array detector (Avondale, PA). Separation of anthocyanins was carried out using a C18 bonded silica column (3µm, 3 x 150mm) from Dionex Inc. (Sunnyvale, CA). Mobile phases consisting of 0.1% trifluoroacetic acid in water (A) and 70% HPLC-grade acetonitrile (B) were prepared and combined in a stepwise gradient over the course of the separation. Gradient conditions were as follows: initial conditions were composed of 90% mobile phase A and 10% mobile phase B; from time 5-10 minutes, the gradient changed to 30% mobile phase B; from 14.5-16 minutes, the gradient changed to 50% mobile phase B; from 20-21 minutes, the gradient was returned to 10% mobile phase B. Anthocyanins were detected in 100µL injections of a 1% solution prepared in mobile phase A at a

temperature and flow rate of 25ºC and 0.6mL/min respectively, and a wavelength of 520nm over the course of 25 minutes. A post run time of 5 minutes was then observed to clear and equilibrate the column for subsequent runs.

It has been previously reported that pelargonidin-*O*-3-glucoside (callistephin) is the major anthocyanin constituent of strawberry fruit (Baiamonte et a. 2010). This was confirmed by comparing a pure callistephin chloride standard (97% pure, Sigma Aldrich, St. Louis, MO) to the extract (Fig. 3.1). The concentration of callistephin in SAE was then determined by constructing a standard curve using the pure callistephin standard. A 1mg/mL solution was prepared initially in mobile phase A, and then diluted to 3/5, 2/5, 1/5, and 1/10 of the original concentration. Standards were injected individually and run under HPLC conditions as described above to create a linear curve ($R^2 \geq 0.990$) of peak area vs. concentration.

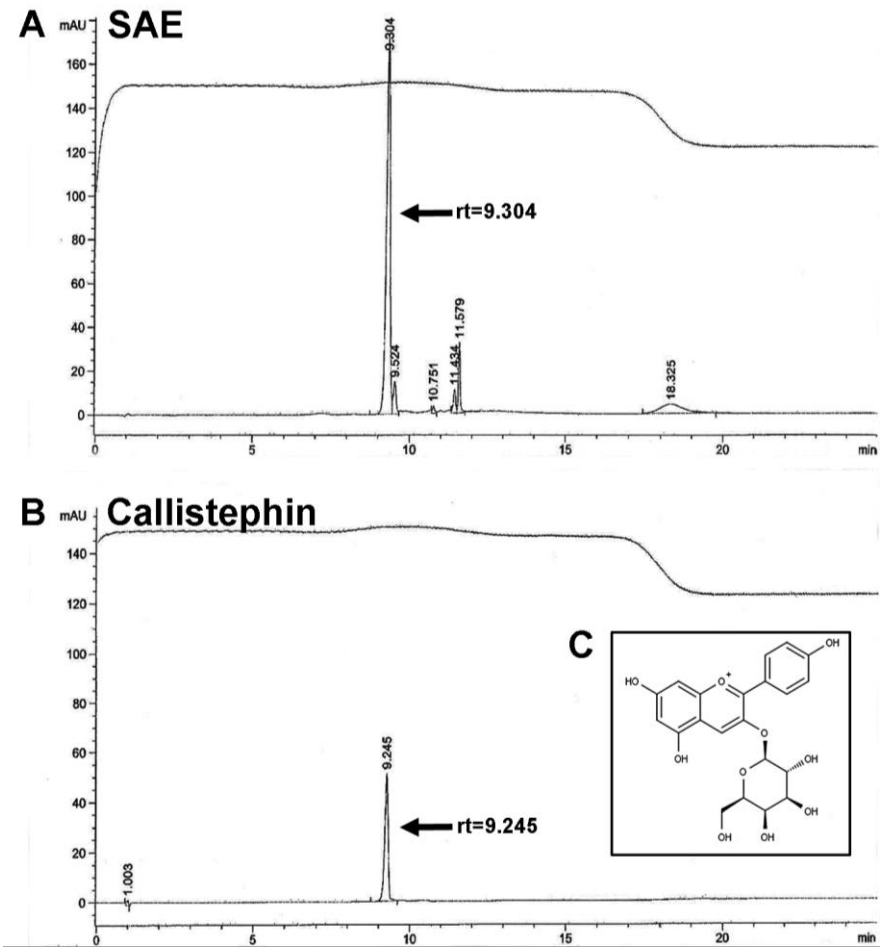

Figure 3.1. Representative HPLC chromatograms of an anthocyanin-enriched extract from strawberries (SAE) and pure callistephin standard. Purified anthocyanin extract from freeze-dried strawberry powder was analyzed by HPLC with UV/Vis detection at 520nm *(A)* and compared to a pure callistephin standard *(B)* for peak identification of the major anthocyanin constituent. Callistephin appears at rt=9.304min for SAE and rt=9.245min for the pure standard. *C,* Inset shows the molecular structure of callistephin.

3.3.2 hSOD1^{G93A} Mouse Model of ALS

All procedures were performed in accordance with a protocol approved by the institutional animal care and use committee (IACUC) at the University of Denver. FVB/NJ mice harboring a human transgene coding for a mutated form of SOD1 with a glycine to alanine substitution at position 93 were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained at the University of Denver animal facility under a standard 12 hour night and day cycle with food and water provided *ad libitum*. Genotyping to identify transgenic mice was carried out by a third party company, Transnetyx Inc. (Cordova, TN).

Mice were divided into three groups of 9 mice each. These groups consisted of non-transgenic wild type (WT) littermate controls, untreated hSOD1^{G93A} littermate controls, and hSOD1^{G93A} mice receiving SAE supplementation. Approximately equal numbers of males and females composed each group. Mice were age and sex-matched to ensure an accurate comparison of clinical indices of disease onset and survival across the three groups. Mice receiving SAE supplementation were dosed twice daily by oral gavage with 0.25mL of a 5% SAE solution prepared in sterile drinking water beginning at the presymptomatic age of 60 days-old. This dose equated to 2mg/kg/day of the primary anthocyanin constituent, callistephin, assuming an average body mass of 25g. SAE supplementation was provided until mice reached the end-stage of disease and were euthanized.

3.3.3 Clinical Tests

Mice were assessed for signs of disease onset daily beginning at 60 days of age. It was determined that mice had experienced disease onset upon the appearance of hind limb trembling or the inability to extend one or both hind limbs when suspended by the tail. Onset was not determined in a blinded fashion; however, onset was evaluated by at least two investigators independently in order to reduce subjectivity.

Mice were also evaluated for grip strength in the hind limbs using the paw grip endurance (PaGE) test as described by Weydt et al. (2003). Briefly, mice were placed on top of a standard wire cage lid, and given a few seconds to acclimate. The lid was then inverted in one smooth motion to prompt the mouse to grip the wire with both its fore and hind limbs. Care was taken to avoid jostling the lid during inversion, which might have caused the mouse to fall. Once inverted, the lid was held suspended a few inches above the bench top and a stopwatch was used to determine the time it took for the mouse to release its grip on the wire lid with its hind limbs after becoming fully inverted. Time was stopped the moment the mouse's hind limbs detached from the cage, or after 30s with no detachment. This time was recorded as latency to fall with a maximum achievable score of 30s. Mice were given three scored attempts, and final scores are reported as the average of the three scores ± standard error of the mean (SEM) for each time point. This testing regimen was performed twice weekly. Time points correspond to the age of the animal at the time that testing was performed, and are reported as a range of five days due to the fact that multiple sets of animals having slightly different ages were tested

concomitantly. Animal weight was also assessed at the time of PaGE testing, immediately following the three scored attempts for each mouse.

Throughout testing, mice were monitored and assessed daily for the point at which end-stage of the disease was reached. End-stage was defined as the point at which a mouse placed on its side failed to right itself to a sternal position within 20s. When it was determined that a mouse had reached end-stage, the animal was immediately euthanized by inhaled isoflurane overdose and secondary decapitation.

3.3.4 Tissue Preparation and Immunohistochemistry

For analysis of disease pathology, a separate set of mice from those used to assess onset and survival was euthanized at 105 days of age, the time point at which the greatest difference in clinical indices of disease was observed. After euthanasia the thoracicolumbar portion of the spinal column, containing the lumbar spinal cord, and gastrocnemius muscle were isolated. Tissue was immediately frozen in optimal cutting temperature (OCT) compound to prepare it for cryosectioning and stored at -80°C until sectioning took place.

For spinal column, 18μm coronal sections were cut, collecting every fourth tissue section onto the surface of Fisherbrand Superfrost coated slides (Thermofisher Scientific, Rockford, IL) to ensure an accurate representation of the entire lumbar region of the spinal cord. Tissue was then fixed at room temperature for forty minutes using 4% paraformaldehyde. Once fixed, tissue was incubated at room temperature in blocking buffer, containing 5% (w/v) bovine serum albumin (BSA) and 0.2% triton-X100 in

phosphate buffered saline (PBS). Primary antibody to glial acidic fibrillary protein (GFAP; Abcam, Cambridge, MA) was then prepared as a 1:250 dilution in PBS containing 0.2% triton-X100 and 2% BSA (w/v) respectively. Tissue sections were then incubated in primary antibody overnight at 4°C. Tissue was next washed five times with PBS to remove unbound primary antibody. FITC-conjugated secondary donkey-anti-rabbit antibody (Jackson Immunoresearch Laboratories, West Grove, PA), was then prepared at a 1:250 dilution (v/v) in PBS containing 0.2% triton-X100 and 2% BSA (w/v) and Hoechst nuclear stain (Sigma-Aldrich, St. Louis, MO) at a 1:500 dilution to detect GFAP primary antibodies and to label nuclei, respectively. Sections were incubated with secondary antibodies for 1h at room temperature, then washed five times in PBS. Slides were then sealed with cover slips using mounting medium composed of a 1:3 of PBS to glycerol (v/v) and p-phenylenediamine (Sigma-Aldrich, St. Louis, MO) to preserve fluorescence. Tissue was then imaged using a Zeiss Axiovert-200M epi-fluorescence microscope to capture a single image of each ventral horn using equal exposure times for all tissue sections. At least 13 ventral horns per animal were imaged and analyzed for fluorescence intensity using Adobe Photoshop CS4 software by determining the average green fluorescence intensity of pixels in the ventral horn region and multiplying the intensity by the total number of pixels that were measured. This analysis was done on four animals for each of the three treatment groups.

For gastrocnemius muscle, 18μm longitudinal sections were cut, collecting every third tissue section on Superfrost coated slides to gain an accurate representation of the size and health of neuromuscular junctions (NMJs). To accomplish this, muscle tissue

was fixed for 40min in 4% paraformaldehyde, and then incubated for 1h in blocking buffer as described above. A 5μg/mL solution of α-bungarotoxin (BTx) conjugated to Alexa Fluor® 594 (ThermoFisher Scientific Inc., Rockford, IL) was then prepared in blocking buffer containing Hoechst nuclear stain at a dilution of 1:500, and sections were incubated for 1h at room temperature. Muscle sections were then washed five times with PBS and sealed with glass cover slips as described above. NMJs were imaged and assessed for size using Adobe Photoshop CS4 software. Size was measured by determining the total area occupied by individual NMJs, and a complete distribution of sizes was created for each of the three treatment groups. At least 50 NMJs per animal were analyzed. NMJs were considered healthy if their morphology was observed to be large and complex, while fragmented and small NMJs were considered to be unhealthy, reflecting a lack of innervation and muscle atrophy (Rudolf et al. 2014). This analysis was done on three animals per each of the three treatment groups.

3.3.5 Nissl Staining for Spinal Motor Neurons

Coronal spinal column sections of 18μm each were cut, and every fourth tissue section was collected on Superfrost coated slides. Tissue sections were fixed for 40min in 4% paraformaldehyde, and then washed with PBS. Subsequently, tissue was prepared by incubation in 70% ethanol for 3min, followed by incubation in 95% ethanol for 3min, and finally two incubations in 100% ethanol for 3min and 5 min respectively, changing the ethanol between each incubation. Slides were then rinsed briefly in deionized water several times. A 1% cresyl violet solution (Ambion, Foster City, CA) was then prepared

in deionized water and applied to the slides for approximately 30s. Slides were rinsed with deionized water several times, and then de-stained to differentiate neuronal cell bodies. To de-stain, tissue sections were dipped briefly into 70mM acetic acid solution. This process was repeated until white space between stained cells was observed under a bright field microscope. Once sections were sufficiently de-stained, the slides were rinsed with deionized water several times, and then incubated sequentially in solutions of 70%, 95%, and 100% ethanol as described above. Slides were then sealed using cover slips and mounting medium composed of a 1:3 (v/v) of PBS to glycerol, and imaged under bright field. Images of the entire ventral horn were captured, and every Nissl-positive cell body was measured and grouped into a category based on its length along its longest axis using Adobe Photoshop CS4 software. These categories included neurons that were <15µm, neurons that were 15-20µm, neurons that were 20-25µm, and neurons that were >25µm in length. Any neurons that were 20µm in size or larger were considered viable motor neurons (Guo et al. 2013); however the data for neurons that were at least 25µm in size was also analyzed as a more stringent evaluation of motor neuron presence and viability. This analysis was performed on at least 13 ventral horns per animal with three animals per each of the three treatment groups.

3.3.6 Statistical Analysis

Histological analyses were performed on at least three animals from each treatment group. Differences in GFAP fluorescence intensity, Nissl-stained cell number, and NMJ size were assessed using one-way analysis of variance (ANOVA) with *post hoc*

Tukey's test to compare differences between groups. Kaplan-Meier curves were analyzed using a log rank test. PaGE and weight data were analyzed using one-way ANOVA with a *post hoc* Tukey's test for each time point. For all analyses, differences were statistically significant when $p<0.05$.

3.4 Results

3.4.1 SAE Delays Disease Onset and Extends Survival in the hSOD1^{G93A} Mouse Model of ALS

We first examined the therapeutic benefit of anthocyanin supplementation in the hSOD1^{G93A} mouse model of ALS by evaluating its impact on disease onset and progression. Mice were treated by oral gavage with an anthocyanin-enriched extract beginning at 60 days of age. This age is considered presymptomatic, as hSOD1^{G93A} mice on an FVB background typically experience disease onset at approximately 90 days of age, although gait abnormalities in hSOD1^{G93A} mice have been reported as early as 8 weeks of age (Mancuso et al. 2011, Ross et al. 2014). Transgenic hSOD1^{G93A} mice receiving SAE treatment displayed a dramatic delay in disease onset with a mean onset of 109.4 ± 2.2 days of age (Fig. 3.2A). In comparison, transgenic mice that did not receive SAE treatment experienced a mean age of onset of 92.6 ± 1.5 days of age. In addition to this delay in disease onset, transgenic hSOD1^{G93A} mice treated with SAE showed a statistically significant extension in survival (Fig. 3.2B). SAE-treated hSOD1^{G93A} mice reached end-stage at an average age of 139.0 ± 2.7 days of age whereas untreated hSOD1^{G93A} mice demonstrated an average lifespan of 127.7 ± 3.0 days of age.

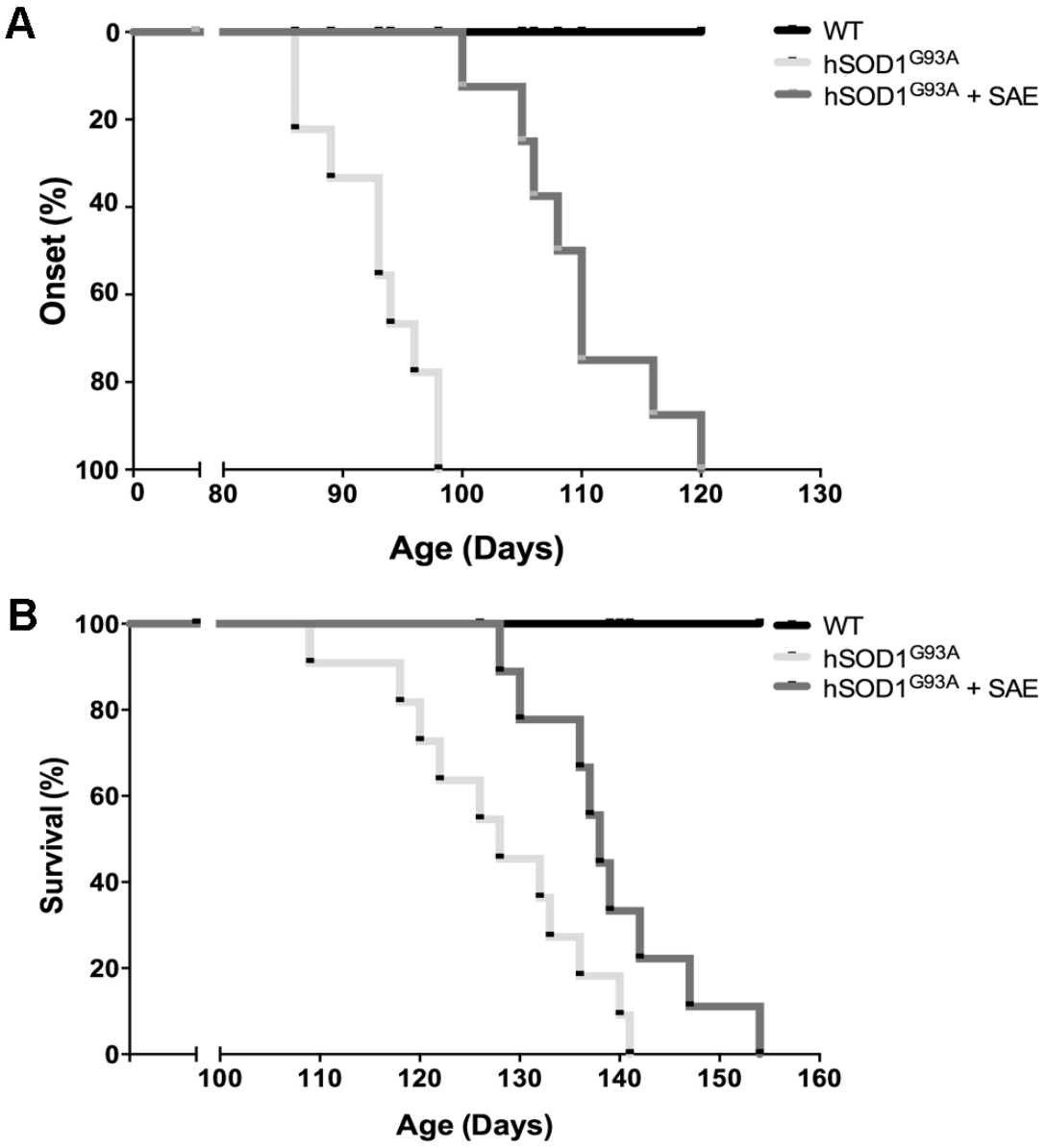

Figure 3.2. Presymptomatic administration of SAE delays disease onset and extends survival in the hSOD1^{G93A} mouse model of ALS. *A,* Administration of SAE beginning at 60 days of age significantly delayed clinical onset of disease in comparison to untreated hSOD1^{G93A} controls. Curves are significantly different as determined by a Mantel-Cox Log Rank test ($p<0.001$, n=9). *B,* Administration of SAE as in *(A)* significantly extended survival of hSOD1^{G93A} mice in comparison to untreated hSOD1^{G93A} controls. Curves are significantly different as determined by a Mantel-Cox Log Rank test ($p<0.01$, n=9).

3.4.2 SAE Preserves Hind Limb Grip Strength in the hSOD1^{G93A} Mouse Model of ALS, But Does Not Affect Body Weight

We next assessed the functional benefit of SAE treatment by evaluating grip strength and body weight loss in hSOD1^{G93A} mice. Deficits in muscle strength and coordination were assessed twice weekly by the PaGE wire hang test. Hind limb grip strength was measured by allowing the mouse to hang from an inverted wire cage lid until its hind limbs detached from the wire. Mice receiving SAE supplementation showed a significant preservation of grip strength between 85 and 124 days of age when compared to hSOD1^{G93A} mice that did not receive treatment (Fig. 3.3A). However, although muscle strength and function were maintained for a longer period of time throughout disease progression, SAE treatment did not significantly affect declines in body weight when compared to untreated hSOD1^{G93A} controls (Fig. 3.3B).

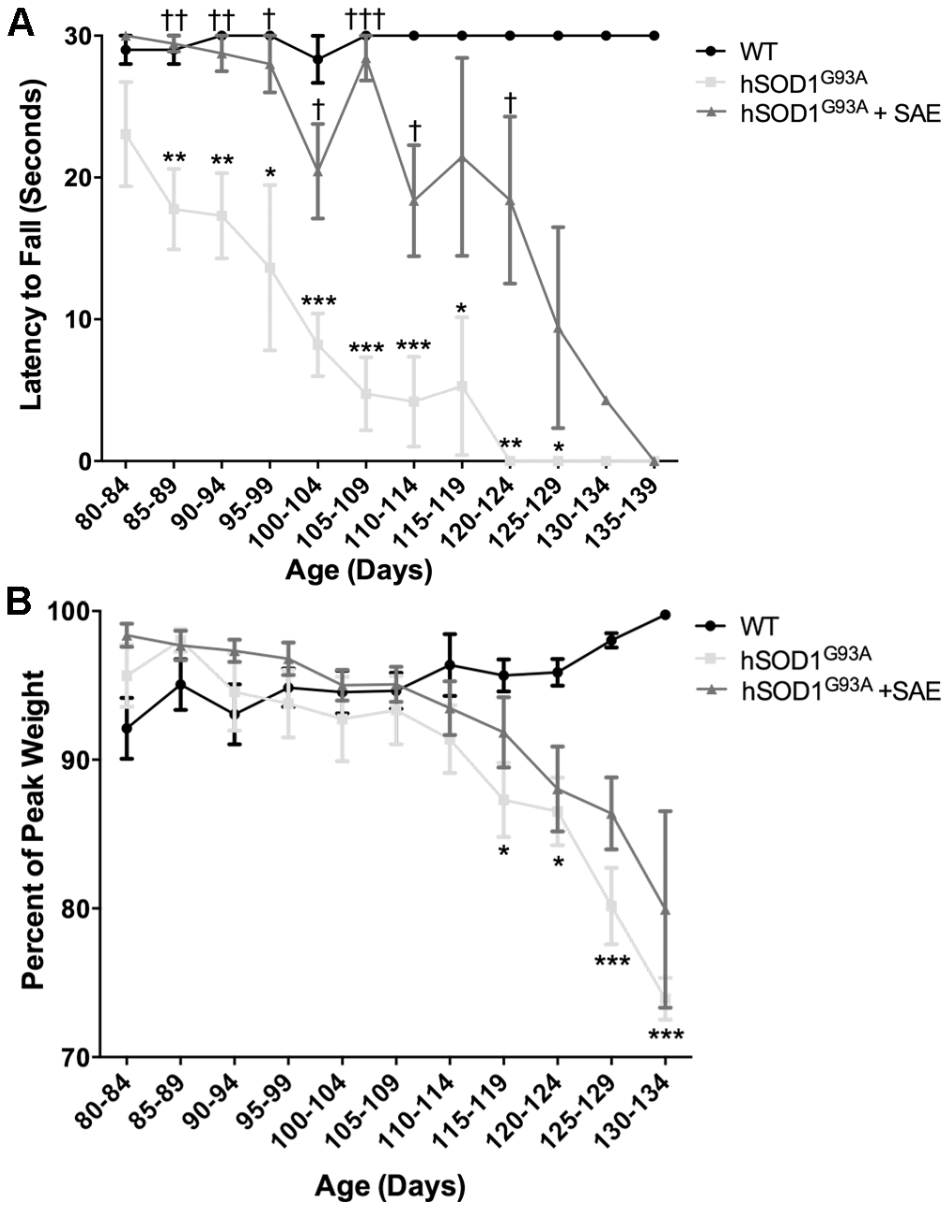

Figure 3.3. Presymptomatic administration of SAE preserves hind limb grip strength in the hSOD1^{G93A} mouse model of ALS. *A,* Paw grip endurance (PaGE) testing. PaGE testing was conducted twice weekly beginning at 80 days of age. Each mouse received three scored attempts for each time point, expressed as latency to fall. *B,* Body weight of WT and hSOD1^{G93A} mice. Body weight was measured twice weekly immediately following PaGE testing, and is expressed as the percent of peak body weight for each time point. All data are expressed as mean ± SEM for each time point, n=8. *** indicates $p<0.001$, ** indicates $p<0.01$ and * indicates $p<0.05$ in comparison to non-transgenic wild type (WT) controls. ††† indicates $p<0.001$, †† indicates $p<0.01$ and † indicates $p<0.05$ in comparison to untreated hSOD1^{G93A} controls. All data were analyzed using one-way ANOVA with a *post hoc* Tukey's test for each time point.

3.4.3 hSOD1^{G93A} Mice Show a Trend Towards Decreased Numbers of Spinal Motor Neurons That is Prevented by SAE

Given the significant clinical benefits produced by treatment with SAE in hSOD1^{G93A} mice, we next assessed the effects of anthocyanin supplementation on histopathological indices of disease. Since motor neuron death is known to underlie ALS, we first evaluated the effects of SAE on preserving motor neuron populations in lumbar spinal cord. As the greatest difference in clinical manifestations of the disease was observed at approximately 105 days of age (see Fig. 3.3A), we selected this time point to evaluate spinal cord tissue for various markers of disease pathology.

Mice were sacrificed at 105 days of age and spinal cord tissue was collected for further analysis. Sections of lumbar spinal cord were Nissl stained to visualize neuronal cell bodies and the number of viable alpha motor neurons in the ventral horns were quantified by measuring each cell body and counting the number of neurons that were either >20μm in size or those that were >25μm in size, a more stringent evaluation of the viable motor neurons. While no statistically significant differences were observed in the total number of motor neurons between the three groups for either size, untreated hSOD1^{G93A} mice showed a trend towards a decreased motor neuron population (Fig. 3.4). This trend was reversed by treatment with SAE, which restored motor neuron numbers to levels comparable to those observed in WT controls in both size categories (Fig. 3.4B, C).

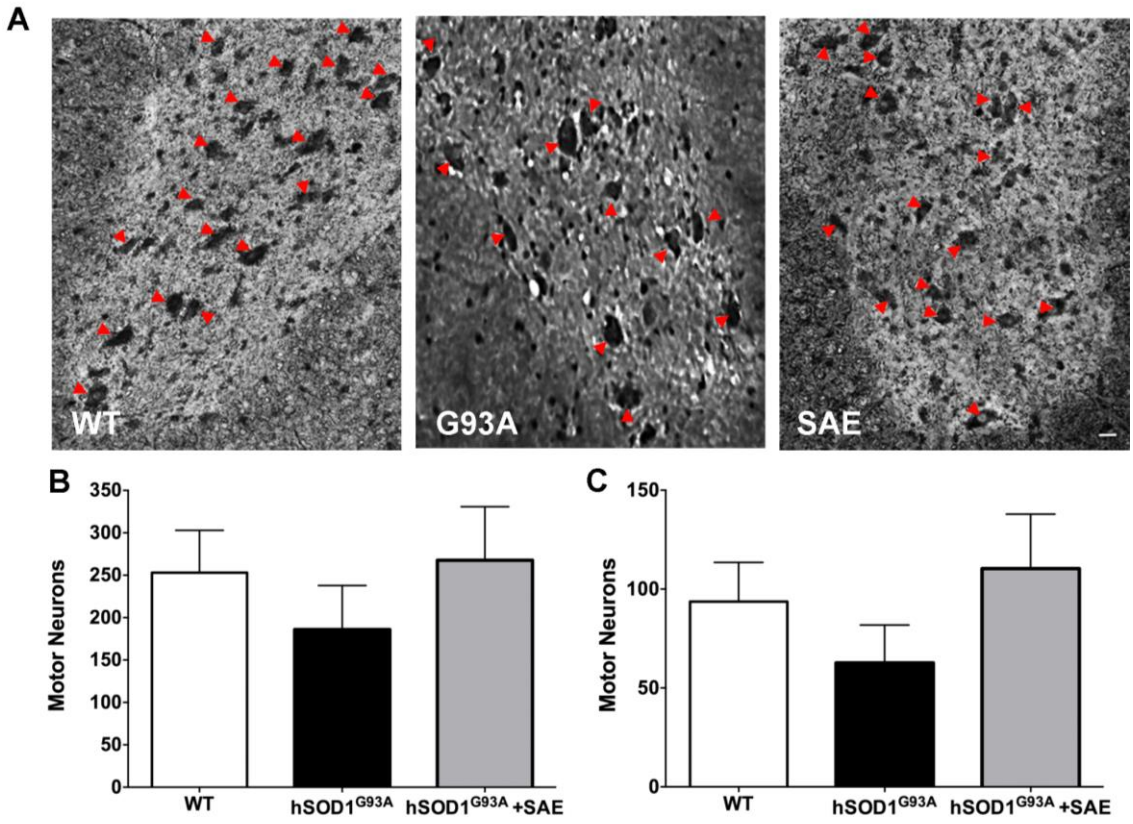

Figure 3.4. Presymptomatic administration of SAE shows a trend towards preserving motor neurons in spinal cord tissue in the hSOD1^{G93A} mouse model of ALS. *A,* Representative images of ventral horns in lumbar spinal cord isolated from non-transgenic wild type controls (WT), untreated hSOD1^{G93A} controls (G93A), and hSOD1^{G93A} mice treated with SAE (SAE). Tissue was isolated at 105 days of age and Nissl stained to identify neuronal cell bodies. Nissl-positive cells were measured individually along their longest axis. Cells were considered viable alpha-motor neurons if they were >20µm in size, indicated by the red arrows. Scale bar =20µm. *B,* Quantitative assessment of Nissl-positive neurons >20µm in size. *C,* Quantitative assessment of Nissl-positive neurons >25µm in size. Data are expressed as mean ± SEM. Motor neurons were counted in at least 13 ventral horns per animal, n=3 mice per group. All quantitative data were analyzed using one-way ANOVA with a *post hoc* Tukey's test.

3.4.4 SAE Reduces Reactive Gliosis in hSOD1^{G93A} Spinal Cord Tissue

As glial-mediated inflammation is thought to be a major contributor to ALS disease progression and motor neuron death, we next examined lumbar spinal cord tissue for the presence of reactive astrocytes by GFAP staining. WT controls displayed very little evidence of reactive astrogliosis in the ventral horns; however, untreated hSOD1^{G93A} mice demonstrated a dramatic increase in the number of astrocytes observed with GFAP staining (Fig. 3.5A). This increase was almost entirely attenuated by treatment with SAE (Fig. 3.5A). In good agreement with these observations, analysis of the total fluorescence intensity measured as green GFAP fluorescence was significantly enhanced in untreated hSOD1^{G93A} controls. Treatment with SAE mitigated this effect, however, returning fluorescence intensity to approximately that observed in WT controls (Fig. 3.5B).

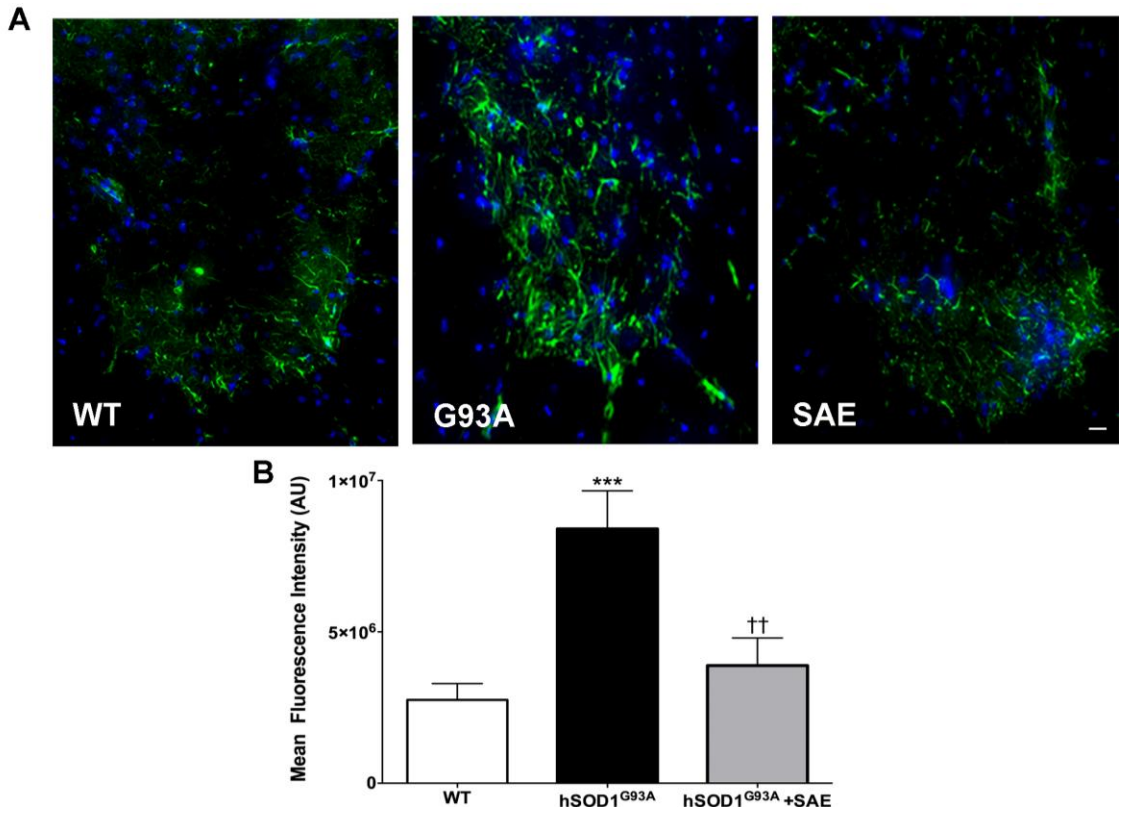

Figure 3.5. Presymptomatic administration of SAE reduces reactive astrogliosis in spinal cord tissue from the hSOD1^{G93A} mouse model of ALS. *A,* Representative images of ventral horns in lumbar spinal cord isolated from non-transgenic wild type controls (WT), untreated hSOD1^{G93A} controls (G93A), and hSOD1^{G93A} treated with SAE (SAE). Tissue was isolated at 105 days of age and stained for GFAP (green) and Hoechst (Blue) to identify reactive astrocytes and nuclei respectively. Scale bar =20μm. *B,* Quantitative assessment of green GFAP intensity. Ventral horns were imaged using equal exposure times and the intensity of GFAP fluorescence was measured. Data are expressed as mean ± SEM. *** indicates $p<0.001$ in comparison to WT controls. †† indicates $p<0.01$ in comparison to untreated mutant hSOD1^{G93A} mice. Measurements were conducted on at least 13 ventral horns per animal, n=4 mice per group. All quantitative data were analyzed using one-way ANOVA with a *post hoc* Tukey's test.

3.4.5 SAE Preserves the Size and Health of Neuromuscular Junctions in hSOD1^{G93A} Gastrocnemius Muscle Tissue

Dying back of motor axons, loss of muscle innervation, and subsequent muscle atrophy are among the first pathological features observed in ALS, becoming apparent even before clinical onset of disease (Pun et al. 2006). Since mice receiving SAE supplementation displayed an impressive retention of grip strength throughout disease progression, but did not display a significant enhancement in motor neuron survival over their untreated counterparts, we hypothesized that effects on grip strength might be explained by preservation of NMJ size and architecture. With this in mind, the area of each NMJ was measured and images were collected to compare morphology. Medial gastrocnemius muscle tissue was isolated from mice at 105 days of age as with spinal cord tissue, and stained with BTx to analyze the size and morphology of NMJs.

WT controls displayed large and elaborate NMJ architecture with smooth margins, indicative of overall muscle health (Fig. 3.6A). In contrast, untreated hSOD1^{G93A} mice possessed NMJs that were much smaller than those observed in WT animals (Fig. 3.6B). Fragmentation of NMJs was also observed, consistent with loss of innervation and muscle atrophy (Fig. 3.6A; Rudolf et al. 2014). This effect was substantially rescued by supplementation with SAE as complex NMJ morphology and size were largely preserved in these animals (Fig. 3.6).

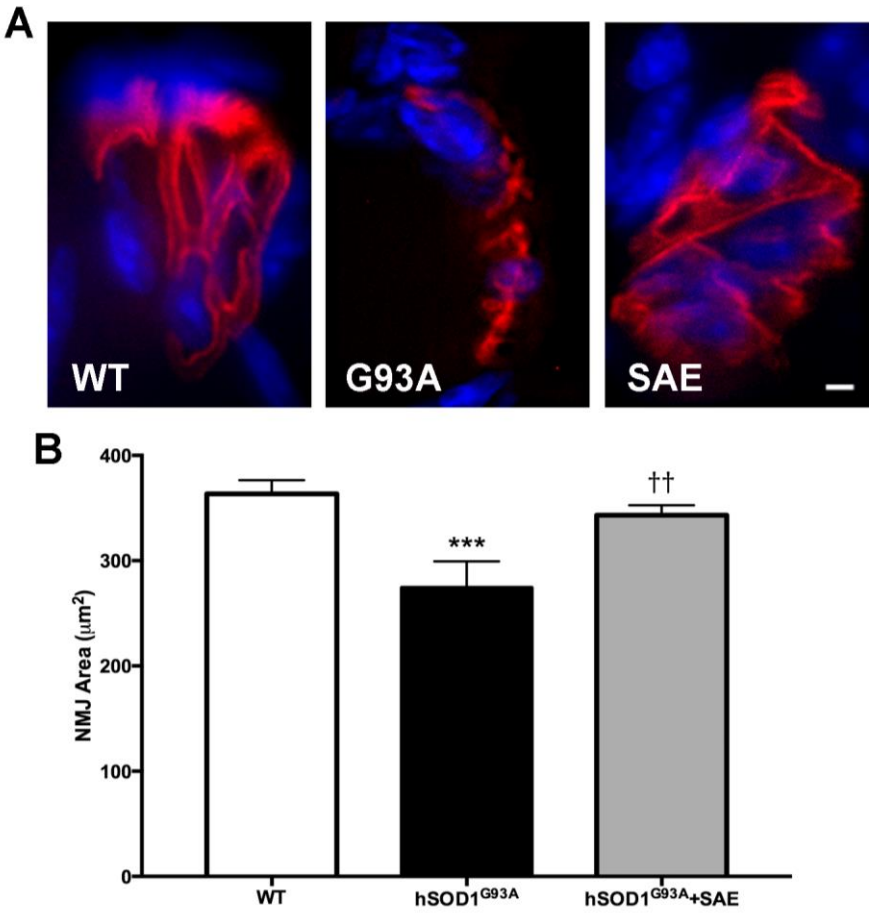

Figure 3.6. Presymptomatic administration of SAE preserves the size and health of NMJs in muscle tissue from the hSOD1^{G93A} mouse model of ALS. *A,* Representative images of neuromuscular junctions in medial gastrocnemius muscle tissue from non-transgenic wild type controls (WT), untreated hSOD1^{G93A} mutant controls (G93A) and hSOD1^{G93A} mice treated with SAE (SAE). Tissue was isolated at 105 days of age and stained with fluorescently labeled α-bungarotoxin (red) and Hoechst (blue) to identify motor endplates and nuclei respectively. Scale bar =10μm. *B,* Quantitative assessment of NMJ size. The area of individual NMJs was measured for 50-150 NMJs per animal. *** indicates $p<0.001$ in comparison to WT controls, and †† indicates $p<0.01$ compared to untreated mutant hSOD1^{G93A} controls, n=3 mice per group. All quantitative data were analyzed using one-way ANOVA with a *post hoc* Tukey's test.

3.5 Discussion

The complex nature of ALS disease pathology has proved to be a great obstacle in the effort to identify new and effective therapeutic strategies. With this in mind, it has become clear that novel treatments are vitally needed to improve the prognosis and quality of life for patients suffering from this insidious disease. Attention has recently turned towards identifying new therapeutic agents that target multiple factors contributing to ALS etiology and progression. Anthocyanins have garnered significant attention in this regard as a potential treatment for neurodegenerative diseases as they have been shown to exert pleiotropic effects on many of the factors thought to underlie these disorders (reviewed by Ross et al. 2013). Therefore, we explored the potential therapeutic benefits of an anthocyanin-enriched extract from strawberries in the preclinical hSOD1^{G93A} mouse model of ALS.

Until now, the potential of anthocyanins as therapeutic agents for ALS has not been examined, although their efficacy in other models of neurodegenerative disease has been described. In the MPTP mouse model of Parkinson's disease, for example, an anthocyanin-rich mulberry extract conferred significant neuroprotection, preserving dopaminergic neurons in the *substantia nigra* and reducing Parkinsonian symptoms (Kim et al. 2010). Similar results were obtained in a study conducted by Roghani et al. (2010), in which the authors demonstrated that oral treatment with pelargonidin, the aglycon form of callistephin, significantly reduced neuronal loss and diminished lipid peroxidation in a 6-hydroxydopamine-lesioned rat model of hemi-Parkinsonism. Positive effects of anthocyanins have also been reported in models of Alzheimer's disease and

aging. Indeed, in the mutant amyloid precursor protein/presenilin-1 (APP/PS1) mouse model of Alzheimer's disease, anthocyanins isolated from bilberry and black currant prevented the deposition of amyloid beta peptides that is characteristic of this disease, and reduced associated cognitive impairments (Vepsäläinen et al. 2013). Similarly, the anthocyanin, cyanidin-O-3-glucoside, was also found to modulate phosphorylation of tau, which is known to form neurofibrillary tangles in Alzheimer's disease, by reducing activation of glycogen synthase kinase-3β following hippocampal injection of amyloid beta peptide (Qin et al. 2013). This reduction in tau phosphorylation was associated with enhanced learning and memory in comparison to rats that did not receive anthocyanin treatment.

In agreement with these prior studies, our results demonstrate that anthocyanin supplementation shows significant preclinical efficacy for the therapeutic treatment of ALS. Administration of SAE at a presymptomatic age significantly delayed clinical disease onset and extended the lifespan of hSOD1^{G93A} mutant mice in comparison to mice that did not receive anthocyanin treatment. In addition, SAE supplementation markedly preserved muscle strength and function in treated mice throughout disease progression as measured by PaGE testing. However, SAE did not affect weight loss in hSOD1^{G93A} animals. This may be due to the fact that anthocyanins are known to enhance weight loss by decreasing fat content; thus SAE-treated mice may have shown a significant reduction in weight, but this could be due to loss of fat while lean muscle is largely preserved in comparison to untreated hSOD1^{G93A} controls (Prior et al. 2008, Tsuda 2008). Further exploration is needed to test this hypothesis. These findings are

highly encouraging in that notable prior studies, such as those assessing the efficacy of vitamin E and Riluzole in this mouse model of ALS, have reported only modest benefits, such as uniquely delaying disease onset or extending lifespan, respectively (Gurney et al. 1996). Furthermore, even when administered at a presymptomatic stage of disease, there is no evidence that either of these treatments is capable of preserving muscle function, which significantly impacts quality of life for ALS patients. Thus, anthocyanin supplementation may be a more efficacious treatment strategy than many of those that have been previously tested.

In addition to improving clinical indices of disease, we also demonstrate that treatment with SAE significantly modulates several aspects of underlying ALS disease pathology. The death of motor neurons is a key pathological factor contributing to ALS etiology. Therefore, strategies aimed at preserving motor neuron populations by mitigating these factors are essential when considering potential therapeutic agents. With this in mind, we first examined the beneficial effects of SAE supplementation on motor neuron survival in the ventral horns of lumbar spinal cord isolated from hSOD1^{G93A} mutant mice. Tissue was collected from mice at 105 days of age, the time point at which the greatest difference in clinical indices of disease were observed between untreated hSOD1^{G93A} controls and mice receiving SAE supplementation. Untreated hSOD1^{G93A} mice demonstrated a trend towards decreased numbers of viable motor neurons in spinal cord tissue, although this decrease did not reach statistical significance. This may be due to the fact that motor neuron viability was assessed at a relatively early time point in disease progression, as marked motor neuron loss is not observed until later stages of the

disease (Dal Canto and Gurney 1994). Nevertheless, it is noteworthy that mice receiving SAE supplementation displayed a trend towards preservation of viable motor neurons in spinal cord tissue, indicating that anthocyanins may display neuroprotective effects in this mouse model of ALS. Indeed, anthocyanins are unique in that they demonstrate a multiplicity of neuroprotective effects, including modulation of neuronal antioxidant status, prevention of perturbances in calcium homeostasis caused by excitotoxic insult, and attenuation of glial inflammation *in vivo* (Dani et al. 2008, Matsunaga et al. 2009, Wang et al. 2010, Poulose et al. 2016, Rehman et al. 2016). These varied effects may contribute to the observed trend towards a positive impact on motor neuron survival.

Reactive astrogliosis plays a prominent role in several neurodegenerative diseases, and its role in ALS specifically is well established as astrocyte-specific knockout of mutant SOD1 has been shown to significantly alter disease progression in mouse models of ALS (Yamanaka et al. 2008, Wang et al. 2011). Here we report that anthocyanin treatment of hSOD1^{G93A} mutant mice with SAE significantly reduced the presence of reactive astrocytes in lumbar spinal cord tissue, which correlates with the trend towards enhanced motor neuron survival observed with SAE administration. This is in good agreement with a very recent study demonstrating that anthocyanins extracted from black soybeans significantly attenuated astrogliosis in cortex and hippocampal tissue isolated from the D-galactose rat model of accelerated aging (Rehman et al. 2016). Furthermore, anthocyanins have been shown to significantly reduce oxidative stress in manganese-treated primary rat astrocytes, a condition that is known to contribute to the

transition of astrocytes from a neuroprotective to a neurotoxic state (da Silva Santos et al. 2014).

Lastly, we examined the impact of anthocyanin supplementation on the health of NMJs in medial gastrocnemius tissue. Since motor neuron loss in untreated hSOD1^{G93A} did not reach statistical significance at 105 days of age, we hypothesized that the marked difference in grip strength observed between untreated hSOD1^{G93A} mice and those supplemented with SAE at this time point was likely due to differences in the health of NMJs in muscles of the hind limbs. Our results show that untreated hSOD1^{G93A} mice displayed a significant reduction in NMJ size and complexity, indicative of muscle atrophy and loss of function, whereas animals treated with SAE displayed NMJs of a size and complexity comparable to WT controls (Rudolf et al. 2014). Thus, it is likely that preservation of healthy NMJs and not preservation of motor neuron viability is responsible for the impressive preservation of grip strength observed in SAE-treated mice at 105 days of age.

While anthocyanins appear to be promising therapeutics, impacting multiple aspects of ALS disease pathology, it is important to note that the results of this study are currently limited by the use of an anthocyanin-enriched extract and the presymptomatic administration of SAE. While widely used and important investigative tools, anthocyanin extracts are poor candidates for potential drug development due to the fact that they contain multiple compounds that may produce synergistic effects *in vivo* and confound the ability of investigators to determine the impact of any specific compound on human health. Additionally, the production of anthocyanin-rich extracts in large quantities would

be costly and difficult to standardize. It is also noteworthy that, presymptomatic administration of treatments in ALS mouse models, though common, represents an unrealistic paradigm for the treatment of human disease, as most ALS patients cannot seek treatment until after clinical disease onset.

Nevertheless, the considerable benefit of SAE administration in the hSOD1^{G93A} mouse model of ALS is highly encouraging, and provides proof of concept that the therapeutic efficacy of both anthocyanin extracts and pure anthocyanin compounds should be further investigated in preclinical models of ALS with administration started at later stages of disease. This is particularly true in light of our recent work demonstrating that different anthocyanin species display differential neuroprotective effects *in vitro*, with anthocyanins such as cyanidin-*O*-3-glucoside displaying broader neuroprotective activity than the closely related anthocyanin callistephin, the major component of SAE (Chapter 2, Section 2.4). With this in mind, it is possible that further preclinical assessment of anthocyanins in the context of ALS and other neurodegenerative diseases may identify anthocyanins with even greater therapeutic benefit than those examined here. Such studies will be of great value moving forward as they have the potential to reveal novel therapeutic agents in addition to defining new mechanisms of action of diverse but closely related nutraceuticals.

CHAPTER FOUR: COMPARISON OF THE DISTINCT NEUROPROTECTIVE AND ANTI-INFLAMMATORY EFFECTS OF THE PHENOLIC ACIDS, PROTOCATECHUIC ACID AND 4-HYDROXYBENZOIC ACID

4.1 Abstract

Neurodegenerative diseases are characterized by the death of specific neuronal populations in the brain and spinal cord. Polyphenols, and in particular, anthocyanins, have been increasingly investigated for their neuroprotective and anti-inflammatory effects in the context of neurodegenerative disease; however the overall bioavailability of many anthocyanins is relatively low. In contrast, phenolic acids, metabolites of many commonly consumed polyphenols, including anthocyanins, have been shown to accumulate in tissue at higher concentrations than those of parent compounds, suggesting that these metabolites may be the bioactive components of anthocyanin-rich diets. We examined the neuroprotective capacity of two common phenolic acids, 4-hydroxybenzoic acid (HBA) and protocatechuic acid (PCA), in primary cultures of cerebellar granule neurons. We demonstrate that both HBA and PCA are capable of mitigating oxidative stress induced by hydrogen peroxide, which is thought to contribute to neuronal cell death in neurodegeneration. Under conditions of nitrosative stress, which occur during inflammation in the central nervous system, only PCA was neuroprotective, despite similar structural characteristics between HBA and PCA. Intriguingly, this trend was

reversed under conditions of excitotoxicity, in which only HBA was neuroprotective. Lastly, we explored the anti-inflammatory activity of these compounds in the BV2 microglial cell line stimulated with lipopolysaccharide. In this model, PCA proved to be an effective anti-inflammatory agent, reducing nitric oxide production and induction of inducible nitric oxide synthase, while HBA had no effect. These data indicate that phenolic acid anthocyanin metabolites possess distinct neuroprotective and anti-inflammatory characteristics that could make them suitable for the treatment of neurodegenerative diseases.

4.2 Introduction

With steady medical advances being made in recent decades, the human population has enjoyed a considerable increase in average life expectancy; however as the population ages, the incidence of neurodegenerative disease has also increased. Indeed, disorders including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS) have all seen a rise in positive diagnoses, prompting extensive research into the etiology and pathology of these diseases (Ingre et al. 2015). Despite these efforts, however, the treatments for these diseases remain scarce and are targeted primarily at reducing symptoms rather than at alleviating causes underlying disease pathology and progression.

Neurodegenerative diseases are characterized by the death of specific neuronal populations within the brain, brain stem and spinal cord, producing significant cognitive and/or motor impairments. Although each disease is uniquely characterized by the type of

neurons that are ultimately affected, the underlying causes of neuronal death are thought to be remarkably similar. These include conditions such as oxidative stress, caused by the buildup of reactive oxygen species (ROS) within the cell that damage vital cellular components such as DNA and proteins, nitrosative stress, caused by damaging reactive nitrogen species (RNS) produced in the central nervous system (CNS) under neuroinflammatory conditions, and excitotoxicity, resulting from overstimulation of neuronal cells by excitatory neurotransmitters, causing massive calcium influx and subsequent activation of cell death signaling cascades (Calabrese et al. 2000, Lin and Beal 2006, Gu et al. 2010, Wang and Qin, 2010, Radi et al. 2014). The identification of agents targeting multiple aspects of neurodegenerative disease pathology, therefore, provides an appealing therapeutic avenue for treating multiple disorders.

In this regard, polyphenols have emerged as promising therapeutic candidates due to their impressive antioxidant, anti-neuroinflammatory, and anti-apoptotic effects (reviewed by Ramassamy, 2006). In particular, anthocyanins, a unique class of flavonoid compounds, show significant potential as a treatment for neurodegenerative disease for these reasons in addition to the observation that they are among the most commonly consumed polyphenolic species around the world (Scalbert and Williamson, 2000). These compounds, responsible for producing the red, blue, and purple pigmentation of many fruits and vegetables, have received significant attention as neuroprotective agents both *in vitro* and *in vivo*; however, the efficacy of these compounds for the treatment of neurodegenerative diseases may be limited by their relatively low bioavailability in the brain following ingestion (Passamonti et al. 2005, Talavera et al. 2005). Indeed,

anthocyanins are rapidly absorbed, and have been shown to accumulate in several areas of the brain; however the levels at which anthocyanins accumulate are extremely low at only ~0.2nmol/g of tissue (Youdim et al. 2003, Andres-Lacueva et al. 2005, Passamonti et al. 2005, Talavera et al. 2005, El Mohsen et al. 2006).

The low levels of circulating anthocyanins are thought to be due in part to extensive metabolism of the parent compounds by gut microflora to form various phenolic acid metabolites (Fleschhut et al. 2006, Woodward et al. 2009, Woodward et al. 2011, Forester and Waterhouse, 2010). This phenomenon has been described by several studies, and it has been reported that incubation of anthocyanin aglycons with gut microflora results in complete degradation of the parent species to form phenolic acids and aldehydes (Woodward et al. 2011). Furthermore, circulating concentrations of phenolic acid metabolites derived from anthocyanin degradation such as protocatechuic acid (PCA), have been observed at up to eight times that of the parent anthocyanins (Azzini et al. 2010). It has also been reported that PCA remains in relevant tissues longer than its parent anthocyanin compounds (Tsuda et al. 1999). Similarly, gallic acid, another phenolic acid metabolite of some anthocyanin compounds, has also been observed to accumulate in brain tissue at high levels with chronic consumption (Ferruzzi et al. 2009). Collectively, these observations have led investigators to suggest that phenolic acids and other anthocyanin metabolites are the bioactive components of anthocyanin-rich diets (Vitaglione et al. 2007). Studies assessing the neuroprotective and anti-inflammatory effects of these compounds are few in number, however, and have focused predominately on only two phenolic acids, PCA and gallic acid. Thus, the

distinct neuroprotective effects of other phenolic acids derived from anthocyanins have not been examined.

We have previously reported that two structurally similar, but distinct anthocyanins, cyanidin-*O*-3-glucoside (kuromanin) and pelargonidin-*O*-3-glucoside (callistephin), display differential neuroprotective effects against a variety of neurotoxic insults. While both callistephin and kuromanin are capable of protecting primary cerebellar granule neurons (CGNs) from mitochondrial oxidative stress and excitotoxicity, only kuromanin is capable of defending neurons from nitrosative stress induced by the nitric oxide donor, sodium nitroprusside (SNP; Kelsey et al. 2011, Chapter 2, Section 2.4). In good agreement with these studies, it has also been reported that PCA and gallic acid display differential abilities to interact with lipid-rich environments and prevent lipid peroxidation dependent upon their relative lipophilicity (Brown and Kelly, 2007). These studies suggest that different anthocyanins, and likely their respective metabolites, may display distinct neuroprotective effects.

Following the work of our previous study on the distinct neuroprotective effects of anthocyanins, we examine the differential neuroprotective effects of two phenolic acids, PCA, the primary metabolite of cyanidin-based anthocyanins, such as kuromanin, and 4-hydroxybenzoic acid (HBA), the primary metabolite of pelargonidin-based anthocyanins, such as callistephin. While the neuroprotective effects of PCA against a variety of stressors such as hydrogen peroxide and SNP have previously been investigated, the neuroprotective abilities of HBA have never been explored, and the anti-inflammatory capabilities of these compounds have not yet been defined (An et al. 2006,

Guan et al. 2006a, Shi et al. 2006, Tarozzi et al. 2007). Moreover, to our knowledge, this is the first study to directly compare the neuroprotective and anti-inflammatory capacities of two distinct, but structurally similar phenolic acids (Fig. 4.1) in order to determine their potential efficacy for the treatment of neurodegenerative disease. Our results demonstrate that PCA and HBA display differential neuroprotective and anti-neuroinflammatory abilities under different neurotoxic conditions, which are described below.

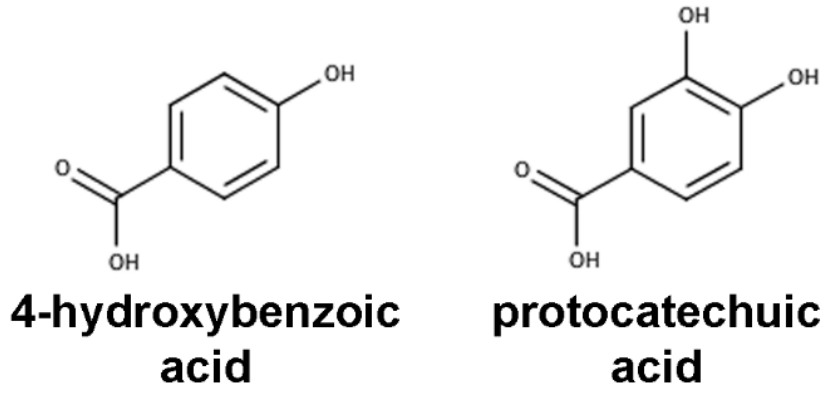

Figure 4.1. Chemical structures of 4-hydroxybenzoic acid (left) and protocatechuic acid (right).

4.3 Methods

4.3.1 Reagents

Protocatechuic acid was purchased from MP Biomedicals (Solon, OH). Potassium chloride, 4-hydroxybenzoic acid, glutamic acid, glycine, lipopolysaccharide (LPS) from *E. coli*, bovine serum albumin (BSA), paraformaldehyde, Hoechst 33258, and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO). Sodium nitroprusside (SNP) was obtained from Calbiochem (San Diego, CA). Basal Medium Eagle's solution, Dulbecco's Modified Eagle's Medium with glucose solution, L-glutamine solution, penicillin/streptomycin solution, and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY) BCA protein assay kits, and Nitric Oxide Assay kits (EMSNO) were obtained from Thermo Scientific (Rockford, IL). Primary antibody to inducible nitric oxide synthase (iNOS) was purchased from Abcam (Cambridge, MA). Primary antibody to β-actin was purchased from Cell Signaling Technology (Danvers, MA). Secondary donkey anti-mouse and donkey anti-rabbit antibodies conjugated to horseradish peroxidase (HRP), polyvinylidene difluoride (PVDF) membranes and ECL reagents were purchased from Amersham Biosciences (Pittsburg, PA).

4.3.2 Cell Culture

CGNs were isolated from 7-day-old Sprague-Dawley rat pups of both sexes (Charles Rivers, location) as previously described (Linseman et al. 2001). Briefly, cells were plated in poly-L-lysine-coated 6-well plates (35mm-diameter) at a density of approximately $4.0x10^6$ cells per well. Cells were maintained in Basal Medium Eagle's

supplemented with 2mM L-glutamine, 25mM potassium chloride, 10% FBS, and penicillin/streptomycin (100U/mL/100μg/mL). After 24h, CGNs were treated with cytosine arabinoside at a concentration of 10μM to inhibit the growth of non-neuronal cells. CGN cultures were then maintained in 10% CO_2 at 37°C for 6-7 days before experiments were performed. This procedure produced cultures that were ~95% pure.

BV2 microglia were maintained in Dulbecco's Modified Eagle's Medium containing 4.5g/L glucose and supplemented with 10% fetal bovine serum and penicillin/streptomycin (100U/mL/100μg/mL). BV2 microglia were maintained in 5% CO_2 at 37°C. For experiments, cells were plated in 6-well plates (35mm-diameter) and allowed to reach 80% confluency before treatment.

4.3.3 Treatment of Cell Cultures

Culture medium was removed and replaced with medium lacking fetal bovine serum to eliminate the neuroprotective effects of serum that might prevent neuronal apoptosis induced by neurotoxic insult. CGNs were then treated with 250μM hydrogen peroxide, 100μM SNP, or 100μM/10μM glutamate/glycine alone or in combination with the indicated doses of either PCA or HBA. CGNs were allowed to incubate under these conditions for 24h prior to assay of neuronal cell death. For all experiments, an untreated control in serum-free medium was used for comparison in assaying cell death.

BV2 microglial cells were treated with LPS at a concentration of 1μg/mL alone or in combination with PCA or HBA at the indicated concentrations in cell culture medium.

Cells were allowed to incubate for 24h prior to cell lysis and assay of nitric oxide production.

4.3.4 Assay of Neuronal Cell Death

Following treatment, CGNs were washed twice with phosphate buffered saline (PBS, pH=7.4) and then fixed for 1h at room temperature with 4% paraformaldehyde. CGNs were then washed again with PBS and stained with Hoechst at a concentration of 10μg/mL to visualize nuclear morphology. Cells were washed with PBS and imaged using a Zeiss Axiovert-200M epi-fluorescence microscope. Five bright field and five nuclear images per well were captured to assess cell death with duplicate wells for each treatment composing one experiment. Cells were counted and scored as either viable or apoptotic based on nuclear morphology using images showing decolorized Hoechst fluorescence. CGNs displaying nuclei with fragmented or condensed morphology were scored as apoptotic, with at least 100 cells per treatment per experiment being scored.

4.3.5 Nitric Oxide Assay

Nitric oxide production was assayed in cell culture medium from BV2 cells following treatment using a nitric oxide assay kit (EMSNO) from Thermo Scientific per the manufacturer's instructions. This kit assays total concentrations of nitrite in solution, one of the major degradation products of nitric oxide, using the Griess method. The concentration of nitrite for each treatment was determined by comparison to a standard curve created using solutions of known nitrite concentrations, with each treatment being performed in duplicate wells.

4.3.6 Cell Lysis and Western Blotting

At 24h after treatment with LPS and either HBA or PCA, whole cell lysates from BV2 cells were prepared for Western blotting essentially as described by Loucks et al. (2006). Protein concentrations were determined for each lysate using a BCA protein assay kit from Thermo Scientific per the manufacturer's instructions. SDS-PAGE was then performed using equal quantities of protein for each treatment, and proteins were then transferred to PVDF membranes for immunodetection. Membranes were then incubated in blocking solution composed of PBS with 0.1% Tween 20 (PBS-T) and 1% BSA for 1h at room temperature to block non-specific binding sites. Membranes were then incubated overnight in primary antibody prepared in blocking solution at 4°C. Following incubation, membranes were washed five times with PBS-T over the course of 25min to remove excess primary antibody. Membranes were then incubated with the appropriate secondary HRP-conjugated antibodies prepared in PBS-T for 1h at room temperature. Excess secondary antibody was removed by washing the membranes with PBS-T as described above, and immunoreactive proteins were detected using enhanced chemiluminescence.

4.3.7 Statistical Analysis

All experiments in both CGNs and BV2 microglia were performed using duplicate wells with each experiment being performed at least three times. Data are represented as the mean ± standard error of the mean (SEM) for the total number of

experiments carried out (n). One-way analysis of variance (ANOVA) with a *post hoc* Tukey's test was used to analyze all data. A p-value of <0.05 was considered statistically significant.

4.4 Results

4.4.1 Both PCA and HBA Protect CGNs from Oxidative Stress Induced By Hydrogen Peroxide

The role of oxidative stress has been extensively documented in several forms of neurodegenerative disease, and indices of oxidative damage have been observed in tissue from the brains and spinal cords of patients with Alzheimer's disease, Parkinson's disease and ALS (Lin and Beal 2006). Hydrogen peroxide (H_2O_2) in particular is thought to play a significant role in these diseases, as proteins such as amyloid beta, implicated in the pathogenesis of Alzheimer's disease, and mutant SOD1, associated with familial forms of ALS, are known to mediate neuronal death in part through enhanced production of this toxic species (Tabner et al. 2005, Ferri et al. 2006). Therefore, we first evaluated the ability of PCA and HBA to protect CGNs from H_2O_2-induced toxicity.

Alone, H_2O_2 induced significant levels of cell death, with approximately 80% of CGNs displaying fragmented and/or condensed nuclei as determined with Hoechst staining, which is consistent with apoptosis (Fig. 4.2A). Furthermore, H_2O_2 treatment caused significant degradation and fragmentation of neuronal processes in addition to shrinkage of neuronal cell bodies, also indicative of cell death. This effect was abrogated by co-treatment of CGNs with either HBA or PCA, which preserved healthy nuclear

morphology comparable to that observed in untreated controls as well as preventing the degradation of neuronal processes (Fig. 4.2A). Quantification of these data revealed that these protective effects are dose dependent, with higher doses of both HBA and PCA offering greater neuroprotection (Fig. 4.2B, C). Moreover, it was shown that PCA protects neurons more efficiently than HBA, demonstrating significant neuroprotection at lower doses than those required to achieve neuroprotection with HBA.

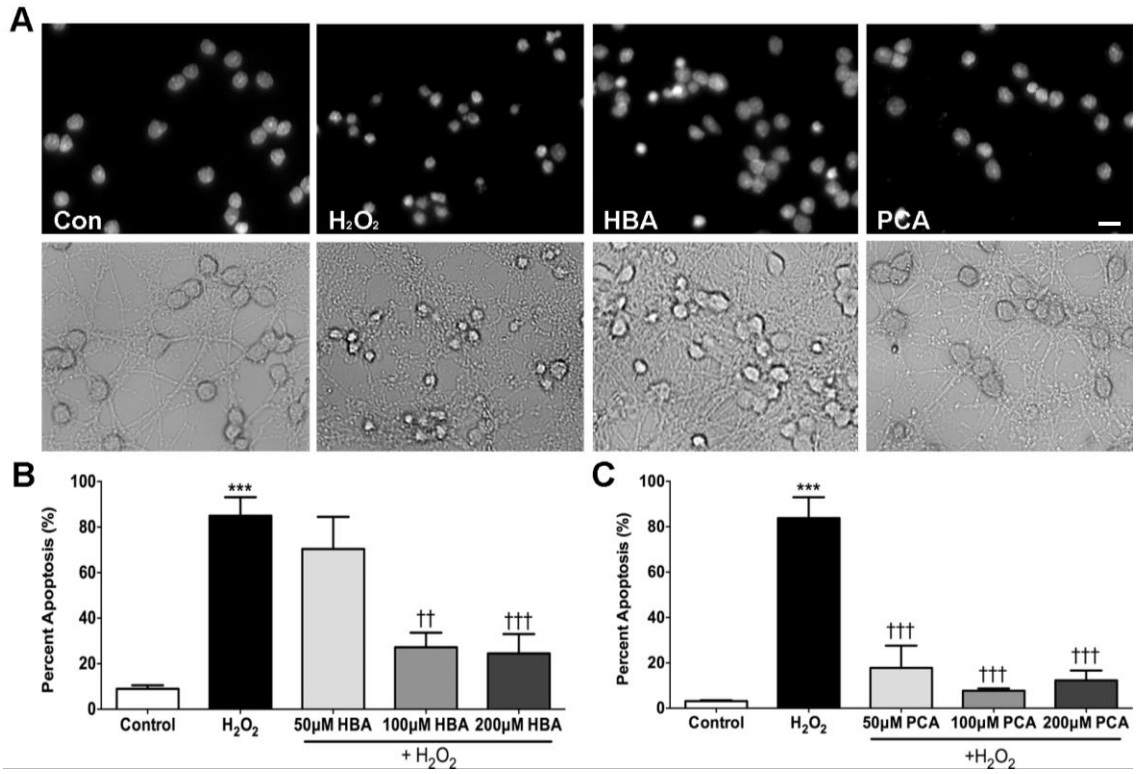

Figure 4.2. Both HBA and PCA protect CGNs from H_2O_2-induced oxidative stress. *A,* Representative images of untreated control CGNs (Con), CGNs treated with H_2O_2 alone (H_2O_2), and CGNs treated in combination with H_2O_2 and either 200μM 4-hydroxybenzoic acid (HBA) or 200μM protocatechuic acid (PCA). Top panels show decolorized Hoechst fluorescence to visualize nuclei. Bottom panels show bright field images to visualize neuronal cell bodies and processes. Scale bar =10μm. *B,* Quantitative assessment of apoptosis in CGNs treated with H_2O_2 alone or in combination with various concentrations of HBA. *C,* Quantitative assessment of apoptosis in CGNs treated with H_2O_2 alone or in combination with various concentrations of PCA. For quantification, nuclear morphology was assessed, and cells displaying condensed or fragmented nuclei were scored as apoptotic. The percent of all total cells that displayed apoptotic morphology was then determined. Data are represented as mean ± SEM for n=3 experiments. *** indicates $p<0.001$ in comparison to untreated controls, and ††† indicates $p<0.001$ and †† indicates $p<0.01$ in comparison to cells treated with H_2O_2 alone by one-way ANOVA with a *post hoc* Tukey's test.

4.4.2 PCA, But Not HBA, Protects CGNs from Nitrosative Stress Induced by Sodium Nitroprusside

Nitrosative stress is a condition similar to oxidative stress caused by a buildup of toxic RNS such as nitric oxide and peroxynitrite, which are capable of causing oxidative damage to many vital cellular components. S-nitrosylation of key cellular proteins involved in protein homeostasis and mitochondrial respiration have been described in the context of ALS and Parkinson's disease respectively indicating a role for RNS in the underlying pathology of these diseases (Walker et al. 2010, Chinta and Andersen, 2011). Nitric oxide and peroxynitrite are produced in abundance during neuroinflammatory responses in the CNS by activated microglia and reactive astrocytes, which have been shown to play a critical role in the etiology and progression of several neurodegenerative diseases (Di Filippo et al. 2010). Thus, compounds that are effectively able to scavenge and detoxify RNS could be of significant therapeutic benefit for the treatment of neurodegeneration. Therefore, we next evaluated the ability of HBA and PCA to mitigate neurotoxicity induced by the nitric oxide donor, SNP.

Treatment of CGNs with SNP alone produced substantial neuronal death, causing extensive nuclear condensation and almost complete obliteration of neuronal processes (Fig. 4.3A). Co-treatment with HBA was unable to attenuate this effect with these cells displaying nuclear morphology and fragmented processes similar to that of cells treated with SNP alone (Fig. 4.3A). In striking contrast to these results, PCA offered complete protection from this insult and preserved healthy nuclear morphology and neuronal processes (Fig. 4.3A). Even at doses as low as 10μM, co-treatment with PCA provided

significant neuroprotection from nitric oxide-induced death, and this effect increased with PCA concentration, while HBA was unable to defend neurons from this insult at any of the doses tested (Fig. 4.3B, C).

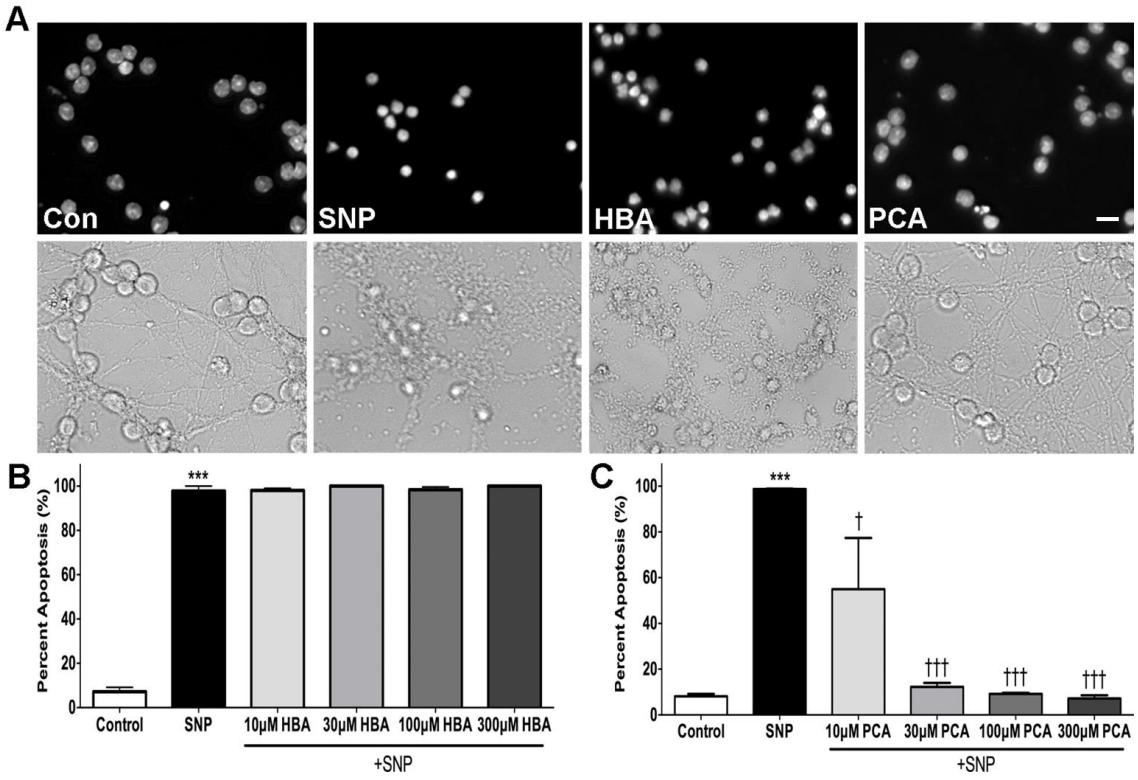

Figure 4.3. PCA, but not HBA, protects CGNs from nitric oxide-induced toxicity. *A,* Representative images of untreated control CGNs (Con), CGNs treated with SNP alone (SNP), and CGNs treated in combination with SNP and either 300μM 4-hydroxybenzoic acid (HBA) or 300μM protocatechuic acid (PCA). Top panels show decolorized Hoechst fluorescence to visualize nuclei. Bottom panels show bright field images to visualize neuronal cell bodies and processes. Scale bar =10μm. *B,* Quantitative assessment of apoptosis in CGNs treated with SNP alone or in combination with various concentrations of HBA. *C,* Quantitative assessment of apoptosis in CGNs treated with SNP alone or in combination with various concentrations of PCA. For quantification, nuclear morphology was assessed, and cells displaying condensed or fragmented nuclei were scored as apoptotic. The percent of all total cells that displayed apoptotic morphology was then determined. Data are represented as mean ± SEM for n=3 experiments. *** indicates $p<0.001$ in comparison to untreated controls, and ††† indicates $p<0.001$ and † indicates $p<0.05$ in comparison to cells treated with SNP alone by one-way ANOVA with a *post hoc* Tukey's test.

4.4.3 HBA, But Not PCA, Protects CGNs from Glutamate-Induced Excitotoxicity

Excitotoxicity is a process specific to neurons in which overstimulation by excitatory neurotransmitters, such as glutamate, causes massive calcium influx from the extracellular space, triggering the activation of a number of cell death cascades, such as calpain-dependent apoptosis (Wang and Qin, 2010). Additionally, disturbances in calcium homeostasis can cause membrane depolarization in mitochondria, resulting in enhanced production of ROS, and release of apoptogenic factors. Considerable evidence for the involvement of excitotoxicity in Alzheimer's disease, Parkinson's disease and ALS has accumulated and suggests that ameliorating excitotoxic effects could be a viable therapeutic approach to treating these diseases (Miguel-Hidalgo et al. 2002, Helton et al. 2008, Parameshwaran et al. 2008, King et al. 2016). Thus, we examined the capacity of HBA and PCA to defend neurons from excitotoxic conditions induced by stimulation with the excitatory neurotransmitter, glutamate.

When treated with glutamate alone, CGNs experienced approximately 50% cell death evidenced by nuclear condensation and fragmentation as well as moderate degradation of neuronal processes (Fig. 4.4A). HBA significantly protected CGNs from glutamate-induced excitotoxicity, preserving nuclear morphology and restoring neuronal processes (Fig. 4.4A). As with H_2O_2, this effect was dose dependent, with the greatest protection occurring with the highest dose of HBA examined in this experiment in direct contrast to PCA (Fig. 4.4B). Under these conditions, PCA displayed no neuroprotective effects, actually displaying a trend towards enhancing neuronal death induced by

glutamate excitotoxicity at higher concentrations, although this trend did not reach statistical significance (Fig. 4.4C).

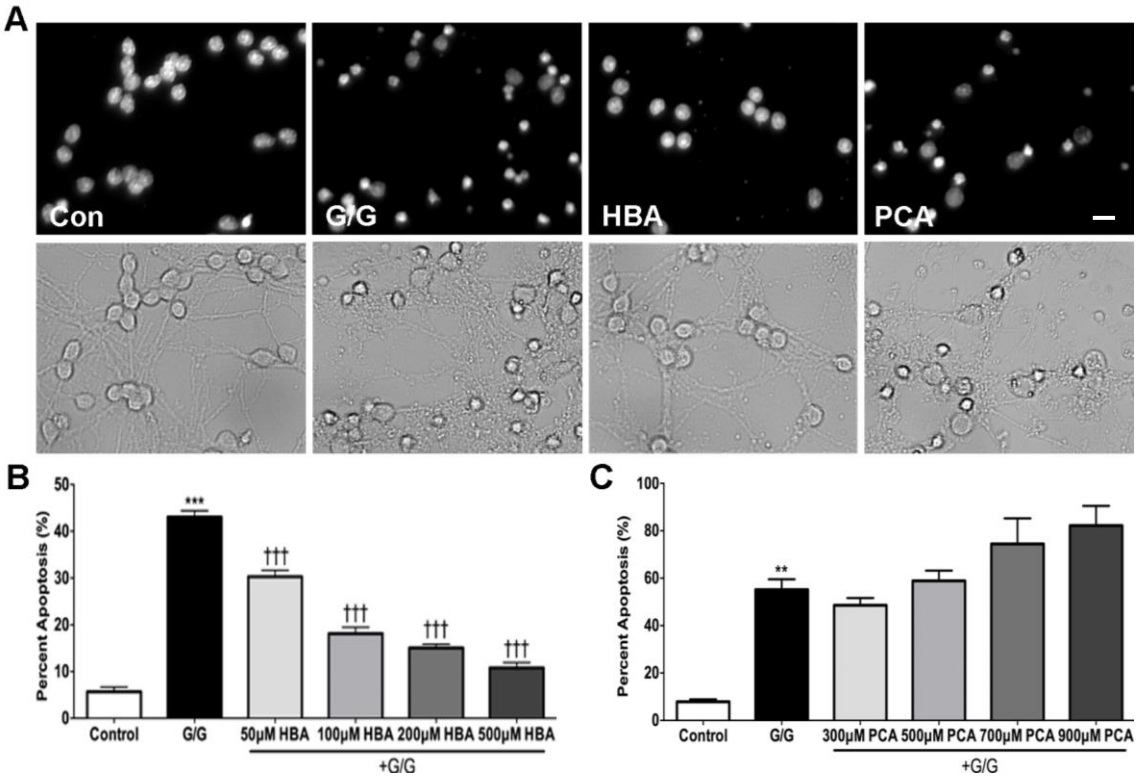

Figure 4.4. HBA, but not PCA, protects CGNs from glutamate-induced excitotoxicity. *A,* Representative images of untreated control CGNs (Con), CGNs treated with glutamate alone (G/G), and CGNs treated in combination with glutamate and either 500μM 4-hydroxybenzoic acid (HBA) or 500μM protocatechuic acid (PCA). Top panels show decolorized Hoechst fluorescence to visualize nuclei. Bottom panels show bright field images to visualize neuronal cell bodies and processes. Scale bar =10μm. *B,* Quantitative assessment of apoptosis in CGNs treated with glutamate alone or in combination with various concentrations of HBA. *C,* Quantitative assessment of apoptosis in CGNs treated with glutamate alone or in combination with various concentrations of PCA. For quantification, nuclear morphology was assessed, and cells displaying condensed or fragmented nuclei were scored as apoptotic. The percent of all total cells that displayed apoptotic morphology was then determined. Data are represented as mean ± SEM for n=3 experiments. *** indicates $p<0.001$ and ** indicates $p<0.01$ in comparison to untreated controls, and ††† indicates $p<0.001$ in comparison to cells treated with glutamate alone by one-way ANOVA with a *post hoc* Tukey's test.

4.4.4 PCA, But Not HBA, Attenuates Lipopolysaccharide-Induced Microglial Inflammation in the BV2 Cell Line

Microglia are the primary mediators of inflammatory immune responses in the CNS. While beneficial in the short term for neuronal protection and repair from foreign invaders, prolonged inflammation of these cells is neurotoxic and has been implicated as a major contributor to the neuronal death underlying neurodegenerative disease (Appel et al. 2011). Indices of microglial inflammation, such as enhanced production of inflammatory cytokines, induction of pro-inflammatory proteins such as COX-2 and inducible nitric oxide synthase (iNOS), and microglial proliferation have been described in Alzheimer's disease, Parkinson's disease, and ALS (Almer et al. 1999, Almer et al. 2001, Hensley et al. 2003, Raoul et al. 2006, Heneka and O'Banion, 2007, Hirsch and Hunot, 2009, Di Filippo et al. 2010). Collectively these studies demonstrate that microglial inflammation is a significant feature of these diseases, making this facet of the neurodegenerative process an appealing therapeutic target. One of the many hallmarks of microglial inflammation is the up-regulation of iNOS and subsequent production of large quantities of nitric oxide (Brown and Neher, 2010). While the ability of potential therapeutic agents to directly scavenge nitric oxide is desirable, as discussed above for SNP, modulation of nitric oxide production and other inflammatory processes is likely to have greater therapeutic benefit overall. Therefore, we assessed the ability of HBA and PCA to alter nitric oxide production and iNOS induction in the BV2 microglial cell line following treatment with LPS.

LPS induced a significant inflammatory response in BV2 microglia marked by a considerable increase in nitric oxide production (Fig. 4.5A, C). This correlated with enhanced expression of iNOS in these cells (Fig. 4.5B, D). Co-treatment of BV2 cells with both LPS and PCA dose-dependently reduced nitric oxide production by a significant amount with corresponding decreases in iNOS protein levels (Fig. 4.5A, B). Co-treatment with HBA and LPS, however, did not produce a significant effect on microglial inflammation, with nitric oxide production and iNOS levels remaining largely unchanged in comparison to microglia treated with LPS alone (Fig. 4.5C, D).

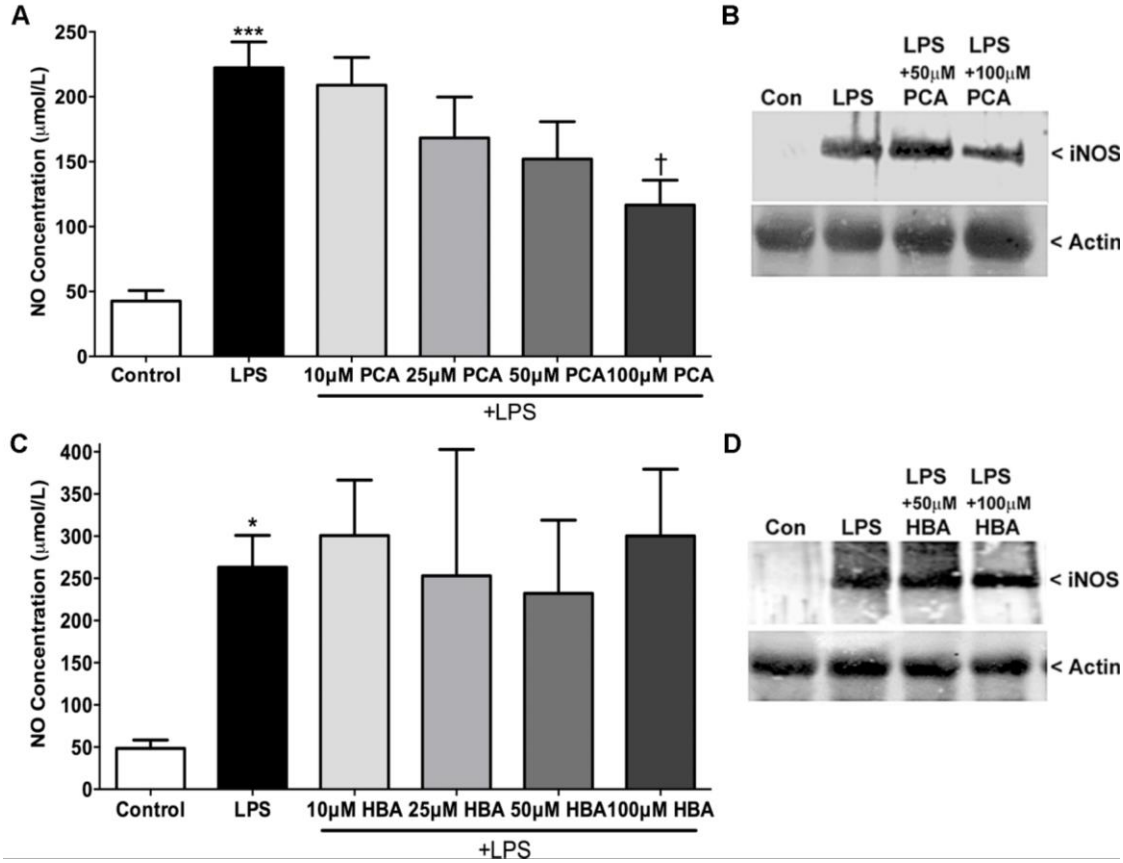

Figure 4.5. PCA, but not HBA, attenuates inflammation induced by LPS in BV2 microglia. *A,* Quantitative assessment of nitric oxide production in untreated, BV2 microglia, and microglia stimulated with LPS alone or in combination with various concentrations of PCA. Nitric oxide production was determined using the Griess method to measure nitrite, a major degradation product of nitric oxide, in cell culture medium incubated with BV2 microglia. *B,* Representative western blots for iNOS (top panel) and β-actin loading control (bottom panel) in whole cell lysates from untreated BV2 microglia, and microglia stimulated with LPS alone or in combination with various concentrations of PCA. *C,* Quantitative assessment of nitric oxide production by BV2 microglia stimulated with LPS alone or in combination with various concentrations of HBA. Nitric oxide production was quantified as in *(A)*. *D,* Representative western blots for iNOS (top panel) and β-actin loading control (bottom panel) in whole cell lysates from untreated BV2 microglia, and microglia stimulated with LPS alone or in combination with various concentrations of HBA. Data are represented as mean ± SEM for n=3 experiments for nitric oxide assay. iNOS blots represent n=1 experiment. *** indicates $p<0.001$, and * indicates $p<0.05$ in comparison to untreated controls, and † indicates $p<0.05$ in comparison to cells treated with LPS alone by one-way ANOVA with a *post hoc* Tukey's test.

4.5 Discussion

Diets rich in anthocyanins and other polyphenols are associated with a myriad of health benefits, including decreased risk of developing cancer, cardiovascular disease, and neurodegenerative disease, particularly Parkinson's disease (Albarracin et al. 2011, Gao at al. 2012). As anthocyanins are known to mitigate multiple facets of the neurodegenerative process thought to contribute to neuronal cell death (reviewed by Ross et al. 2013), this finding is perhaps unsurprising; however current research suggests that it is likely anthocyanin metabolites produced after anthocyanin ingestion that are truly responsible for mediating these positive effects *in vivo*. We have previously compared the neuroprotective abilities of two anthocyanin species, callistephin and kuromanin, against mitochondrial oxidative stress, nitrosative stress, and excitotoxicity, and found that slight structural variances in anthocyanin structure significantly influence the neuroprotective capacity of these compounds against different neurotoxic insults (Kelsey et al. 2011, Chapter 2, Section 2.4). Given these data in conjunction with the observation that phenolic acid metabolites from anthocyanins are likely responsible for mediating the beneficial effects of these compounds *in vivo*, it is equally important to determine if different anthocyanin metabolites display differential neuroprotective functions that may influence their overall effectiveness as potential therapeutic agents in neurodegenerative disease. However, while the neuroprotective capacity of some anthocyanin metabolites has been previously evaluated, a systemic comparison of the effects of phenolic acid metabolites from anthocyanins against a broad range of neurotoxic insults has never been conducted. Here, we evaluated the broad neuroprotective and anti-inflammatory effects

of two phenolic acid metabolites, HBA and PCA, which are derived from pelargonidin-based and cyanidin-based anthocyanins respectively.

Our results demonstrate that HBA and PCA display both similar and distinct neuroprotective effects against several neurotoxic stressors. While both compounds are capable of defending CGNs from H_2O_2-induced oxidative stress, consistent with previous studies conducted with PCA, their protective effects diverge in the context of nitrosative stress and glutamate excitotoxicity (An et al. 2006, Guan et al. 2006a, Shi et al. 2006, Tarozzi et al. 2007). In agreement with prior reports, PCA is a highly effective neuroprotective agent against nitric oxide-induced death following treatment with SNP (An et al. 2006). These results are in remarkable contrast to those observed for HBA, which demonstrate that this compound is incapable of mitigating nitric toxicity to any degree. Our previous work with kuromanin and callistephin, parent compounds of PCA and HBA respectively, also displayed this trend, with kuromanin protecting CGNs to a significant degree from SNP toxicity, while the closely related anthocyanin, callistephin, showed no effect on nitric oxide-induced death (Chapter 2, Section 2.4). This previous work also demonstrated that protection from nitric oxide by kuromanin appeared to be catechol-dependent, a structural feature that both kuromanin and its phenolic acid metabolite, PCA, share, but which is notably lacking from the structure of both callistephin and its metabolite, HBA (Fig. 4.1). Thus, it is very likely that this slight structural difference between HBA and PCA that gives PCA a catechol moiety is responsible for their vastly different neuroprotective abilities against nitric oxide toxicity.

This trend appears to be reversed in the context of excitotoxicity. In CGNs stimulated with glutamate, HBA offered significant neuroprotection, while PCA treatment had no effect on neuronal viability against this insult. This result was unexpected as we have previously shown that both callistephin and kuromanin, two of the parent compounds of these phenolic acids, are potent inhibitors of excitotoxic cell death in CGNs (Chapter 2, Section 2.4). Furthermore, it has been reported that PCA is capable of reducing increases in intracellular calcium concentrations in primary cortical neurons treated with amyloid-beta, which are thought to occur through activation of N-methyl-D-aspartate (NMDA) receptors at the cell surface (Ban et al. 2007, Alberdi et al. 2010). As massive calcium influx through NMDA receptors is also a major feature of glutamate excitotoxicity, it is somewhat surprising that PCA does not protect CGNs from glutamate treatment. The possibility remains, however, that the protective effects of PCA on neuronal viability in the context of amyloid beta toxicity are due to mechanisms other than its ability to attenuate disruptions in calcium homeostasis. These mechanisms could be distinct from those observed under excitotoxic conditions, which could render PCA ineffective for alleviating excitotoxic stress. Alternatively, this could also suggest that the ability of PCA to regulate calcium homeostasis is not sufficient to protect CGNs from glutamate excitotoxicity. This then suggests two possible mechanisms to explain the ability of the closely related HBA to preserve neuronal viability following excitotoxic insult in contrast to PCA. One simple explanation for the differences observed between these compounds is that they may both regulate calcium homeostasis, but HBA may do so more effectively than PCA, which could in turn prevent activation of downstream pro-

apoptotic signaling to a greater extent. Conversely, preservation of neuronal viability by HBA but not PCA could be reliant on the ability of HBA to activate pro-survival or inhibit pro-apoptotic signaling pathways involved in excitotoxic death, an ability that PCA may lack due to structural differences between these compounds. However, since neither PCA nor HBA has been examined for their neuroprotective effects against excitotoxicity until now, further exploration of this topic is needed to define the neuroprotective mechanism of HBA treatment under these conditions.

Finally, we assessed the ability of these compounds to attenuate LPS-induced inflammation in the BV2 microglial cell line. Under inflammatory conditions, we again observed a stark difference between the abilities of HBA and PCA, with PCA acting in an anti-inflammatory capacity, while HBA treatment produced no effect on microglial inflammation. This was assessed by the ability of these compounds to reduce nitric oxide production and induction of iNOS, two hallmarks of microglia-mediated inflammation in the CNS (Brown and Neher, 2010). PCA effectively reduced nitric oxide production, which correlated with reduced protein levels of iNOS; however HBA had no effect on either nitric oxide production or iNOS expression. Intriguingly, another anthocyanin metabolite, gallic acid, has also been shown to reduce microglial inflammation in a similar manner, although the effects of PCA and HBA in BV2 microglia have not been evaluated until now. Reductions in inflammation by gallic acid were reported to prevent microglial-mediated toxicity in co-cultured neurons, suggesting that some anthocyanin metabolites may mediate neuroprotective effects *in vivo* through amelioration of microglial inflammation (Kim et al. 2011). Despite these observations, the mechanism

underlying PCA's ability to attenuate neuroinflammatory responses in microglia is unknown; however, reductions in iNOS expression suggest that PCA may modulate regulators of the gene encoding iNOS, such as nuclear factor-κB. This phenomenon has been well-established in BV2 microglia treated with extracts rich in anthocyanins, which may share this ability with some phenolic acid metabolites. A lack of protection by HBA also suggests this mechanism as differences in structural features between HBA and PCA may allow them to regulate different signaling pathways, as suggested above. Nevertheless, further study is needed to confirm or refute this hypothesis.

Collectively, these data highlight the intriguing neuroprotective and anti-inflammatory differences in anthocyanin metabolites, while also indicating some limitations of these compounds to mitigate various factors involved in the neurodegenerative process. For example, while both HBA and PCA may effectively target toxicity induced by ROS, only PCA targets microglial inflammation and nitrosative stress, and only HBA targets excitotoxicity. However, the ideal therapeutic candidate for the treatment of neurodegeneration would target all four of these aspects of disease. It is interesting to note, then, that PCA and HBA display complimentary effects, suggesting that combination treatment with both of these agents may be an effective therapeutic strategy to target a broader range of factors involved in the neurodegenerative process, which could produce greater therapeutic effects in preclinical models of neurodegenerative disease than administration of either compound alone. This is a particularly appealing strategy in light of several recent *in vivo* studies demonstrating that supplementation with PCA alone is an effective therapeutic treatment in mouse models of

Parkinson's disease, Alzheimer's disease, and D-galactose-induced accelerated aging (Zhang et al. 2010, Tsai and Yin, 2012, Song et al. 2014). Finally, it is important to note that although they are discussed here in the context of anthocyanin metabolism, both HBA and PCA can be produced by the metabolism of other polyphenolic species. As the neuroprotective and anti-inflammatory effects of these compounds appear to be dose-dependent, identifying parent compounds that can be metabolized to produce greater concentrations of PCA and HBA than anthocyanins *in vivo* could be an effective strategy for identifying new therapeutic candidates for testing in preclinical models of disease.

In summary, both HBA and PCA display distinct neuroprotective effects *in vitro* in primary CGNs, suggesting that these compounds warrant further exploration both alone and in combination to further define their neuroprotective and anti-inflammatory mechanisms under diverse stress conditions. Moreover, further exploration of these compounds in preclinical models of disease is warranted as both compounds target multiple aspects of neurodegenerative disease pathology. In particular, the use of HBA and PCA in combination could be of great therapeutic potential owing to the diverse and complimentary neuroprotective effects of these compounds against oxidative stress, nitrosative stress, and excitotoxicity, as well as the anti-neuroinflammatory effects of PCA.

CHAPTER FIVE: PROTOCATECHUIC ACID, AN ANTHOCYANIN METABOLITE, EXTENDS LIFESPAN AND PRESERVES GRIP STRENGTH IN THE HSOD1^{G93A} MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

5.1 Abstract

Amyotrophic lateral sclerosis (ALS) is a malicious neurodegenerative disease characterized by the progressive loss of motor neurons in the brain and spinal cord. However, despite significant advances in our understanding of ALS pathology, the underlying cause of the disease remains unknown, and treatments for the disease are scarce. In recent years, abundant evidence demonstrating the complex nature of the disease has emerged, and several factors are thought to contribute to motor neuron death. Thus, therapeutic strategies for this disease have begun to focus on the identification of agents that target multiple facets of disease pathology. In particular, nutraceutical compounds, such as anthocyanins, have shown great promise as neuroprotective and therapeutic agents in preclinical models of neurodegenerative disease, stroke, and aging, which share common pathological features with ALS. However, the bioavailability of anthocyanins *in vivo* is relatively low, leading investigators to suggest that the beneficial effects of anthocyanin supplementation are likely due to the activity of anthocyanin metabolites, and not the parent compounds themselves. We have previously shown that an anthocyanin-enriched extract from strawberries produces significant therapeutic

benefits in the transgenic G93A mutant human Cu, Zn-superoxide dismutase ($hSOD1^{G93A}$) mouse model of ALS; however the therapeutic potential of direct supplementation with anthocyanin metabolites has never been examined. Therefore, we evaluated the effects of supplementation with a major anthocyanin metabolite, protocatechuic acid (PCA), on ALS disease progression. We show that supplementation with PCA beginning at disease onset significantly extends lifespan and preserves skeletal muscle function in hind limbs of $hSOD1^{G93A}$ mice. These preliminary data suggest that PCA may be a viable therapeutic agent for the treatment of ALS and related diseases, although further research is needed to evaluate the effects of PCA administration on histopathological indices of disease.

5.2 Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult onset motor disease and is marked by progressive weakness, loss of motor function and coordination, muscle atrophy, and paralysis. These characteristics of the disease are due to the death of motor neurons in the brain, brain stem and spinal cord, leading to a loss of muscle innervation. ALS is inevitably fatal, and patients usually experience death 2-5 years following their diagnosis due to respiratory failure. While our understanding of ALS pathogenesis has grown, and genetic causes for familial forms of the disease have been identified, a cause for the vast majority of sporadic ALS cases is unknown. This has made designing effective therapeutic agents for ALS exceedingly difficult; however several factors contributing to the death of motor neurons in this disease have been

identified, and may represent effective therapeutic targets for future treatment development. Specifically, the roles of oxidative stress, nitrosative stress, excitotoxicity, and neuroinflammation are currently topics of intense focus in ALS research due to accumulating evidence that these factors all contribute to motor neuron death and disease progression (Carri et al. 2015, Hooten et al. 2015, King et al. 2016). Oxidative stress is a prominent feature of ALS and is thought to occur due to a combination of enhanced reactive oxygen species production by damaged mitochondria and reductions in endogenous antioxidant defenses (Carri et al. 2015). Accumulation of free radicals and other oxidative species then causes significant damage to vital cellular components such as lipid membranes and DNA. Similarly, nitrosative stress occurs when reactive nitrogen species generation is elevated, particularly under conditions of neuroinflammation, and contributes to nitrosative modification of important cellular components, such as those involved in protein homeostasis (Walker et al. 2010, Hooten et al. 2015). Neuroinflammation is mediated primarily by microglia surrounding motor neurons, which become chronically enflamed and adopt a neurotoxic phenotype as ALS progresses in which they secrete large quantities of pro-inflammatory cytokines, chemokines, and nitric oxide (Hooten et al. 2015). Lastly, excitotoxicity occurs as a result of over-stimulation with excitatory neurotransmitters, particularly glutamate, causing calcium influx into post-synaptic cells, activation of apoptotic signaling pathways, and ultimately neuronal death (King et al. 2016). Enhanced glutamate release, and impaired glutamate clearance from the synaptic cleft are observed ALS patients and mouse models of ALS, indicating a role for this phenomenon in ALS pathogenesis (Rothstein et al.

1995, Fray et al. 1998, Sasaki et al. 2000, Allaman et al. 2011, Milanese et al. 2011, Giribaldi et al. 2013). With the complexity of this disease in mind, a growing consensus has emerged that effective therapeutic strategies for disease treatment should target multiple facets of ALS disease pathology.

Several trials have been conducted with agents, such as vitamin E, an antioxidant, olesoxime, a mitochondrial protectant, and Riluzole, an anti-glutamatergic, that target one significant aspect of disease pathology, and while these potential therapeutics showed promising results in preclinical trials, their effects in the clinic were only modest at best (Graf et al. 2005, Miller et al. 2012, Lenglet et al. 2014). Indeed, of these agents, only Riluzole has shown any true clinical efficacy, although its overall effect on disease progression is very small, extending lifespan by 2-3 months (Miller et al. 2012). The failure of these treatments highlights the need for new therapeutic agents that display pleiotropic effects and target multiple disease pathways.

Recently, anthocyanins have emerged as novel therapeutic agents in preclinical studies of neurodegeneration, and clinical studies of mild cognitive impairment. These flavonoid compounds, found abundantly in many types of berries, have garnered significant attention for several reasons. First and foremost, anthocyanins are among the most commonly consumed polyphenolic species in the western diet, making them an appealing dietary supplement for a variety of health disorders (Scalbert and Williamson, 2000). Secondly, anthocyanins have been shown to have impressive neuroprotective and anti-inflammatory properties that make them well-suited as potential therapeutics for neurodegenerative diseases (reviewed by Ross et al. 2013). *In vivo* studies have revealed

that anthocyanins display significant antioxidant and anti-inflammatory activities in models of carbon tetrachloride-induced cognitive impairment and LPS-induced inflammation, respectively (Dani et al. 2008, Wang et al. 2010). Moreover, several studies have examined the neuroprotective effects of these unique compounds in the context of NMDA-induced excitotoxicity as well as models of Parkinson's disease and Alzheimer's disease and discovered that anthocyanins are capable of limiting oxidative damage and activating pro-survival signaling pathways in these paradigms (Matsunaga et al. 2009, Kim et al. 2010, Roghani et al. 2010, Shih et al. 2010, Qin et al. 2013, Vepsäläinen et al. 2013). However, while these studies are promising, they do not take into account that the relative bioavailability of anthocyanins *in vivo* is quite low due to the rapid metabolism of these compounds following ingestion (Fleschhut et al. 2006, Woodward et al. 2009, Woodward et al. 2011, Forester and Waterhouse, 2010). This has led investigators to speculate that anthocyanins themselves likely contribute very little to the beneficial health effects associated with an anthocyanin-rich diet, suggesting instead that anthocyanin metabolites are responsible for these positive findings (Vitaglione et al. 2007).

Several phenolic acid metabolites are derived from anthocyanins by degradation of the parent compounds by gut microflora (Fleschhut et al. 2006, Woodward et al. 2009, Woodward et al. 2011, Forester and Waterhouse, 2010). Among these, the compound protocatechuic acid (PCA) has begun to garner recent attention as an effective neuroprotective agent. PCA is a prevalent anthocyanin metabolite, and is observed at concentrations up to eight times higher than that of its parent compounds in the blood

following anthocyanin ingestion (Azzini et al. 2010). It has also been shown to remain in tissue for longer periods of time than anthocyanins, suggesting that it may be among the bioactive components of an anthocyanin-rich diet (Tsuda et al. 1999). This is supported by reports that PCA displays potent neuroprotective abilities against oxidative stress induced by hydrogen peroxide and nitric oxide toxicity induced by sodium nitroprusside (An et al. 2006, Guan et al. 2006a, Shi et al. 2006, Tarozzi et al. 2007). We have also shown that PCA possesses anti-inflammatory effects in a microglial cell line treated with LPS, an *in vitro* model of neuroinflammatory microglia, which are observed in ALS (Chapter 4, Section 4.4.4). Furthermore, our work demonstrated that higher doses of PCA offered greater neuroprotection against oxidative and nitrosative stress as well as microglial inflammation, suggesting that direct supplementation with PCA at higher concentrations than those observed following anthocyanin metabolism may be a more effective therapeutic strategy than treatment with the parent compounds (Chapter 4, Section 4.4). It is also important to note that PCA is a major metabolite of other polyphenols, such as procyanidins and other flavonoid species, making it a prevalent component of polyphenol-rich diets. Collectively, these data suggest that PCA supplementation may be a viable therapeutic strategy for the treatment of diseases like ALS for which conditions of oxidative and nitrosative stress, and neuroinflammation are major contributing factors. Thus, we examined the beneficial effects of PCA supplementation on the transgenic G93A mutant human Cu, Zn-superoxide dismutase ($hSOD1^{G93A}$) mouse model of ALS. Our preliminary results indicate that PCA may be a promising therapeutic candidate for further preclinical testing.

5.3 Methods

5.3.1 hSOD1^{G93A} Mouse Model of ALS

All procedures were performed in accordance with a protocol approved by the institutional animal care and use committee (IACUC) at the University of Denver. Mice harboring a human transgene coding for SOD1 with a glycine to alanine substitution at position 93 on an FVB/J background were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were bred on an FVB/NJ background. Breeding and maintenance of all mice took place at the University of Denver animal facility. Mice were kept under a standard 12-hour night and day cycle with chow and water provided *ad libitum.* Mice were genotyped by a third party company, Transnetyx Inc. (Cordova, TN), to identify transgenic animals.

Mice were divided into three groups: non-transgenic wild type controls (WT), untreated hSOD1^{G93A} transgenic mice, and hSOD1^{G93A} mice receiving PCA supplementation (MP Biomedicals, Solon, OH). Each group consisted of seven mice, with roughly equal numbers of males and females in each group. Each set of mice was composed of age and sex-matched littermates to ensure accurate comparison of clinical indices of disease and survival across the three groups. Those mice that received PCA supplementation were dosed once daily by oral gavage with 0.25mL of a PCA solution prepared in sterile drinking water at a dose of 100mg/kg beginning at 90 days of age, which coincides with the average age of disease onset for this model (Ross et al. 2014). Treatment with PCA proceeded until mice had reached end-stage of disease, at which time the mice were euthanized.

5.3.2 Clinical Tests

Hind limb grip strength was assessed using the paw grip endurance (PaGE) test as previously described by Weydt et al (2003). Mice were placed on top of a standard wire cage lid and allowed to acclimate before the lid was inverted in one smooth motion, prompting the mice to grip the wire with their fore and hind limbs. The lid was then suspended a few inches above the bench top, and the time at which mice released their grip on the cage lid with their hind limbs was determined using a stopwatch. Mice were monitored for a maximum of 30s, or until their hind limbs detached from the wire, and this time was recorded as latency to fall. Each mouse was given three scored attempts, and final scores are reported as the average of these attempts ± standard error of the mean (SEM) for each of the indicated time points. PaGE testing was performed twice weekly, and time points correspond to the age of the animals at testing. Time points are represented as a range of five days due to the fact that several groups of mice having slightly different ages were tested concomitantly. Body weight was also measured immediately following PaGE testing for each individual mouse.

For the duration of the study, mice were monitored on a daily basis for signs of progressive motor deficits. When mice reached end-stage of disease, defined as the point at which a mouse placed on its side failed to right itself to a sternal position within 20s, they were euthanized. Euthanasia was performed by exposing the animals to inhaled isoflurane overdose, followed by secondary decapitation and tissue collection.

5.3.3 Statistical Analysis

Kaplan-Meier curves were analyzed using a Mantel-Cox log rank test. PaGE and weight data were analyzed using one-way analysis of variance (ANOVA) with a *post hoc* Tukey's test for each time point. For all analyses, differences were statistically significant when $p<0.05$.

5.4 Results

5.4.1 Administration of PCA at Disease Onset Extends Survival in the hSOD1^{G93A} Mouse Model of ALS

The potential therapeutic efficacy of PCA supplementation was first examined by evaluating its effect on survival in the hSOD1^{G93A} mouse model of ALS. Mice were administered PCA at a dose of 100mg/kg (assuming an average body weight of 25g) beginning at 90 days of age, which coincides on average with the first clinical signs of disease onset (Ross et al. 2014). At this age, hSOD1^{G93A} mice typically display beginning signs of muscle weakness, presenting as trembling in the hind limbs and failure to fully extend one or both hind limbs when the mouse is suspended by its tail (Gurney et al. 1994, Ludolph et al. 2007, Scott et al. 2008). Under this treatment paradigm, mice receiving PCA experienced a dramatic extension in survival over their untreated hSOD1^{G93A} counterparts (Fig. 5.1). Indeed, PCA-treated mice displayed a mean survival of 140.5 ± 4.3 days while untreated hSOD1^{G93A} mice experienced a mean survival of 122.8 ± 2.9 days. While preliminary, these data represent a significant alteration in disease progression in PCA-treated mice.

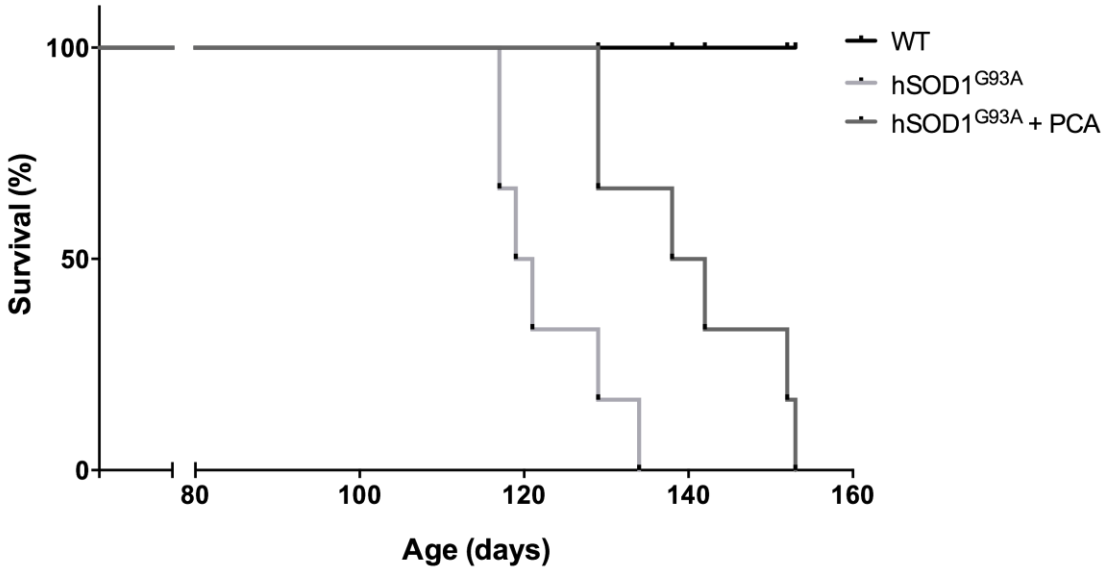

Figure 5.1. Administration of PCA at disease onset extends survival in the hSOD1^{G93A} mouse model of ALS. Administration of PCA beginning at 90 days of age significantly extended the lifespan of hSOD1^{G93A} mice in comparison to untreated hSOD1^{G93A} controls. Curves are significantly different as determined by a Mantel-Cox Log Rank test ($p<0.01$, $n=6$).

5.4.2 Administration of PCA at Disease Onset Preserves Grip Strength, But Does Not Affect Body Weight in the hSOD1^{G93A} Mouse Model of ALS

In addition to studying the effects of PCA on survival, the effects of this compound on hind limb strength and function were also assessed as measures of quality of life. Intriguingly, at 90 days of age, both untreated and treated hSOD1^{G93A} mice already displayed marked reductions in hind limb grip strength in comparison to WT controls. However, as the study progressed, untreated hSOD1^{G93A} mice experienced continuing decline in grip strength as assessed by PaGE testing while mice receiving PCA maintained grip strength comparable to that observed at 90 days of age well into the disease course (Fig. 5.2A). Specifically, mice treated with PCA showed significantly higher grip strength than untreated hSOD1^{G93A} mice from 95-114 days of age before experiencing declines in hind limb muscle strength and function.

Declines in body weight beginning at disease onset are a major characteristic of the hSOD1^{G93A} mutant mouse model of ALS, so we next evaluated the ability of PCA to attenuate weight loss in this model. Both untreated hSOD1^{G93A} mice and mice receiving PCA supplementation displayed steady reductions in body weight as the disease progressed in comparison to WT controls (Fig. 5.2B). No significant differences were observed in the rate of body weight loss between the two groups of hSOD1^{G93A} mice.

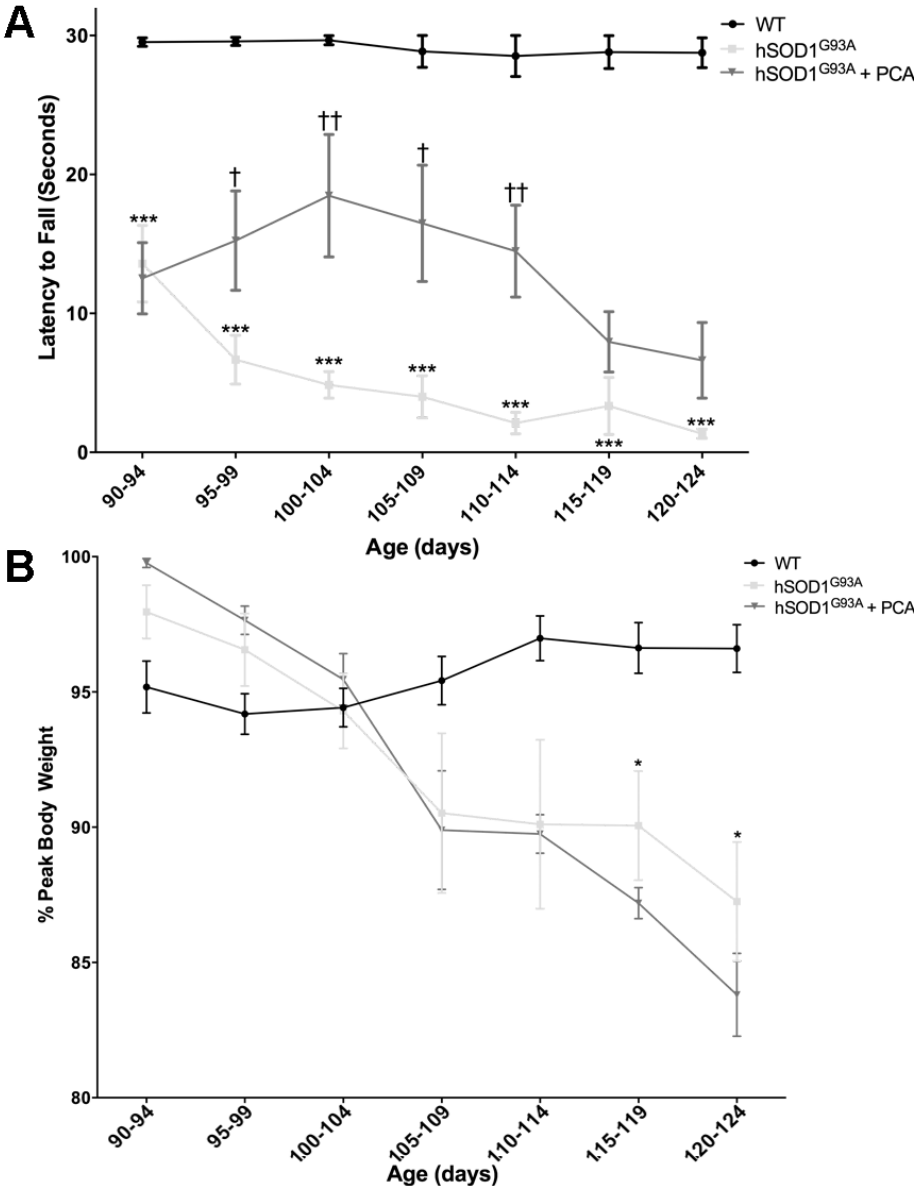

Figure 5.2. Administration of PCA at disease onset preserves hind limb grip strength in the hSOD1^{G93A} mouse model of ALS. *A,* Paw grip endurance (PaGE) testing. PaGE testing was conducted twice weekly beginning at 90 days of age. Each mouse received three scored attempts for each time point, expressed as latency to fall. *B,* Body weight of WT and hSOD1^{G93A} mice. Body weight was measured twice weekly immediately following PaGE testing, and is expressed as the percent of peak body weight for each time point. All data are expressed as mean ± SEM for each time point, n=7. *** indicates $p<0.001$, and * indicated $p<0.05$ in comparison to non-transgenic wild type (WT) controls. †† indicates $p<0.01$ and † indicates $p<0.05$ in comparison to untreated hSOD1^{G93A} controls. All data were analyzed using one-way ANOVA with a *post hoc* Tukey's test for each time point.

5.5 Discussion

Oxidative and nitrosative stress as well as neuroinflammation are major features of ALS disease pathology, and have been described in both patient tissue and several animal models of this disease. In particular, it has been noted that there are significant reductions in endogenous anti-oxidant defenses, such as glutathione, in patient blood and cortex, while lipid peroxidation and oxidative DNA damage are enhanced (Bogdanov et al. 2000, Simpson et al. 2004, Babu et al. 2008, Weiduschat et al. 2014). Furthermore, it has been reported that S-nitrosylation of key proteins involved in protein homeostasis, such as protein disulfide isomerase, are elevated in patient spinal cord tissue, highlighting the role of nitrosative damage in this disease (Walker et al. 2010). Elevations in oxidative damage are thought to be due in part to mitochondrial dysfunction, causing large increases in the production of toxic species such as superoxide, an assertion supported by the observation that mitochondrial redox status can be altered by mutant forms of SOD1 (Ferri et al. 2006). Moreover, activity of pro-oxidant enzymes such as NADPH-oxidase-2 (NOX-2) are increased in microglia from spinal cord tissue in both ALS patients and a mouse model of the disease, suggesting that oxidative damage in motor neurons can be attributed to both cell-autonomous and non-cell-autonomous mechanisms (Wu et al. 2006).

Markers of neuroinflammation are also observed at elevated levels in cerebrospinal fluid from ALS patients and spinal cord tissues from ALS mouse models. Pro-inflammatory cytokines such as tumor necrosis factor-α and Fas ligand have both been observed at elevated levels, as have pro-inflammatory enzymes such as

cyclooxygenase-2 and inducible nitric oxide synthase (iNOS; Almer et al. 1999, Almer et al. 2001, Hensley et al. 2003, Raoul et al. 2006). It has also been reported that glia-specific knockout of mutant SOD1 in mutant mouse models of ALS, which attenuates the inflammatory phenotype of microglia and astrocytes, is neuroprotective, providing compelling evidence for the role of neuroinflammation in this disease (Beers et al. 2006, Boilée et al. 2006, Yamanaka et al. 2008, Wang et al. 2011).

In light of the growing consensus that targeting multiple facets of ALS disease pathology may provide better clinical benefits than targeting any one aspect in particular, we previously explored the therapeutic efficacy of treating hSOD1^{G93A} mice with an anthocyanin-enriched extract from strawberries (Chapter 3). Anthocyanins are best known for their high levels of intrinsic antioxidant activity, which has been used in part to explain their impressive neuroprotective effects, though recently, their anti-inflammatory activity in microglia has also been described (Lau et al. 2007, Shih et al. 2007, Zafra-Stone et al. 2007, Zhu et al. 2010, Hwang et al. 2011, Carey et al. 2013, Poulose et al. 2012, Jeong et al. 2013). In good agreement with these data, we found that anthocyanins displayed a marked trend towards preserving motor neuron viability in spinal cord tissue from hSOD1^{G93A} mice. This trend correlated with significant reductions in the presence of reactive astrocytes in spinal cord and preservation of healthy neuromuscular junctions in hind limb muscle tissue. Moreover, mice receiving anthocyanin supplementation experienced both a significant delay in disease onset and corresponding extension in lifespan (Chapter 3, Section 3.4). This supports the idea that targeting multiple aspects of disease pathology, particularly oxidative stress and

neuroinflammation, is an efficacious therapeutic strategy. However, it is of utmost importance to note that the beneficial effects of anthocyanins *in vivo* are now generally believed to be due to activity of anthocyanin metabolites rather than the parent anthocyanin compounds. Indeed, one report indicated that up to 73% of cyanidin-based anthocyanins are converted to PCA shortly after human ingestion, and circulating concentrations of this compound far exceeded that of the parent anthocyanins (Vitaglione et al. 2007). Additionally, our previous work with PCA has indicated that this compound retains some of the neuroprotective and anti-inflammatory activity of its parent compounds *in vitro*, and that these effects are dose dependent (Chapter 4, Section 4.4). Thus, it was of great interest to assess the therapeutic benefit of direct PCA supplementation in the hSOD1^{G93A} mouse model to determine if this compound shows similar or greater preclinical efficacy than its parent anthocyanin compounds.

Our preliminary data indicate that administration of PCA at disease onset significantly extends the lifespan of hSOD1^{G93A} mice in comparison to their untreated counterparts. Additionally, we found that PCA administration significantly preserved hind limb grip strength well into the disease course, consistent with a slowing in disease progression. However, PCA supplementation did not significantly impact alterations in body weight throughout the disease course. It has been reported that parent anthocyanins are capable of promoting weight loss in this manner, although the influence of PCA on weight has not been examined (Prior et al. 2008, Tsuda 2008). Thus, it is possible that the lack of effect on body weight by PCA could be due to reductions in overall body fat while lean muscle mass is preserved to some extent. While preliminary, these data are

very promising for the preclinical efficacy of this compound for treating ALS and support previous findings demonstrating that PCA is an effective therapeutic agent in other preclinical models of neurodegenerative disease and aging. In the MPTP mouse model of Parkinson's disease, for example, PCA was shown to have neuroprotective effects on dopaminergic neurons in the *substantia nigra* (Zhang et al. 2010). Similarly, in mouse models of aging and Alzheimer's disease, PCA was shown to decrease markers of oxidative damage in addition to reducing expression of several pro-inflammatory factors, likely associated with astroglial or microglial activity (Tsai and Yin, 2012, Song et al. 2014). Together with our data, these findings suggest that PCA may show similar results to that of its parent anthocyanin compounds on motor neuron survival and proliferation of reactive astrocytes when histopathological indices of disease are measured, although further study is needed to confirm these hypotheses.

Another important factor when evaluating the potential therapeutic efficacy of PCA is the timing of administration. While it is common place to perform preclinical studies in the hSOD1^{G93A} mouse model of ALS using a presymptomatic dosing strategy so as not to rule out promising therapeutic candidates (Leitner et al. 2009), this paradigm does not translate well to clinical practices for ALS patients. Because the large majority of ALS patients cannot seek treatment until the onset of symptoms, administration of therapeutic agents is often impossible at presymptomatic stages of the disease. Thus, it is becoming a more common practice to test potential therapeutic candidates at disease onset, as this methodology more closely resembles clinical practices. However, many therapeutic candidates that show preclinical benefits in the hSOD1^{G93A} mouse model at

presymptomatic stages of the disease are not effective when administered at disease onset. As such, it is very encouraging to note that PCA shows a relatively large effect on survival and grip strength when administered at disease onset, although these data are preliminary. In the future it will be of considerable interest to determine if this effect retains significance as more mice are assessed. It will also be of significant value to determine the effects of PCA supplementation on histopathological indices of ALS to further characterize the neuroprotective mechanisms of this compound.

CHAPTER SIX: CONCLUSIONS AND FUTURE DIRECTIONS

The use of anthocyanins and their metabolites as therapeutic agents in several preclinical models of neurodegenerative disease and aging has grown substantially in recent years, and yielded promising results for the preclinical efficacy of these compounds. However, a systematic comparison of the neuroprotective effects of anthocyanins and their metabolites has not been conducted until now despite reports that distinct anthocyanins and phenolic acids display significant differences in their ability to act in various biological systems, such as lipid-rich environments and membranes (Youdim et al. 2003, Brown and Kelly, 2007, Faria et al. 2014). Furthermore, although positive results for the use of anthocyanins and their metabolites have been observed in other disease models, their preclinical efficacy in the context of ALS has not been explored until now. Thus, the principle goal of this dissertation was to examine the potential of anthocyanins and their metabolites as therapeutic candidates for the treatment of ALS. To accomplish this task, anthocyanins and anthocyanin metabolites were first examined *in vitro* to establish their neuroprotective characteristics before further evaluation *in vivo* in the hSOD1^{G93A} mouse model of ALS. The major findings of these studies are summarized below.

6.1 Summary of Major Findings

Previous work in our lab studied the neuroprotective effects of two structurally similar but distinct anthocyanin species, callistephin and kuromanin, against mitochondrial oxidative stress induced by inhibition of the pro-survival protein, Bcl-2 (Kelsey et al. 2011). These data demonstrated that both anthocyanins under investigation displayed similar neuroprotective effects under these treatment conditions. Given these encouraging results, we wished to elaborate upon these findings by further characterizing the neuroprotective effects of callistephin and kuromanin against other neurotoxic insults. Our results, detailed in Chapter 2, revealed that while both anthocyanins were able to potently inhibit the neurotoxic effects of glutamate excitotoxicity, only kuromanin was able to protect primary cerebellar granule neurons (CGNs) from nitric oxide toxicity. Further examination of the mechanism underlying this effect revealed that the neuroprotective abilities of kuromanin were dependent upon the catechol moiety located on the B-ring of this anthocyanin, which is notably lacking in the closely related anthocyanin, callistephin. Furthermore, we demonstrate that this effect is dependent on generation of superoxide through auto-oxidation and possible redox cycling of this catechol structure, defining a highly unique and novel mode of neuroprotection for kuromanin and its related compounds.

Since we observed positive neuroprotective effects in both our previous studies, and those detailed in Chapter 2, we next assessed the therapeutic benefit of anthocyanin treatment in the hSOD1^{G93A} mouse model of ALS. This study was the first to appraise the therapeutic efficacy of anthocyanin supplementation in a model of ALS, and yielded

highly encouraging results. Mice that received anthocyanin supplementation beginning at a presymptomatic age displayed a significant delay in disease onset with a corresponding extension in survival. Furthermore, testing of hind limb grip strength was used as a measure of muscle function, and by extension, quality of life. Testing showed that supplementation with anthocyanins preserved grip strength well into the disease course in comparison to hSOD1^{G93A} mice that did not receive anthocyanins. These findings correlated with the observation that untreated hSOD1^{G93A} mice showed a trend towards decreased numbers of viable motor neurons in addition to a marked increase in the number of reactive astrocytes in lumbar spinal cord tissue. These effects were reversed by treatment with anthocyanins. Lastly, the health of neuromuscular junctions (NMJs) was evaluated. Mice receiving anthocyanin supplementation displayed NMJs with an average size and morphology that was comparable to that of non-transgenic wild type (WT) controls, whereas untreated hSOD1^{G93A} mice experienced marked reductions in the size of NMJs as well as fragmented morphology characteristic of NMJ deterioration. These results suggest that anthocyanins may be effective therapeutic agents for the treatment of ALS.

Nevertheless, reports that the relative bioavailability of anthocyanins *in vivo* is quite low prompted us to consider the hypothesis that the beneficial effects of anthocyanin supplementation are due to the activity of anthocyanin metabolites, rather than the activity of the parent compounds. With this in mind, we next explored the neuroprotective and anti-inflammatory activity of the anthocyanin metabolites, 4-hydroxybenzoic acid (HBA) and protocatechuic acid (PCA), derivatives of callistephin

and kuromanin, respectively. The results of this study, discussed in Chapter 4, demonstrated that, like their parent compounds, HBA and PCA displayed neuroprotective effects that were both similar and distinct. While both HBA and PCA protected primary CGNs from oxidative stress induced by hydrogen peroxide, only HBA was able to defend neurons from glutamate excitotoxicity. In contrast, under conditions of nitric oxide toxicity and neuroinflammation, HBA had no effect while treatment with PCA protected CGNs from nitric oxide generated by a nitric oxide donor, and reduced levels of nitric oxide and inducible nitric oxide synthase (iNOS) expression in BV2 microglial cells. These results were intriguing from the standpoint that these two compounds display complimentary effects against these various neurotoxic insults. Thus, although both are effective neuroprotective agents individually, HBA and PCA may display even greater neuroprotective effects when administered in tandem.

As anthocyanin metabolites proved to be effective neuroprotective agents *in vitro*, we next sought to determine the preclinical value of these compounds in the treatment of ALS, which is discussed in Chapter 5. In particular, PCA was selected for further study as this compound is one of the most common anthocyanin metabolites generated in mammalian systems, and it has been shown to have a positive impact in animal models of Parkinson's disease, Alzheimer's disease, and aging (Zhang et al. 2010, Tsai and Yin, 2012, Song et al. 2014). Preliminary data collection revealed that hSOD1^{G93A} mice receiving PCA supplementation beginning at disease onset displayed a marked extension in survival. Moreover, PCA treatment appeared to halt declines in hind limb grip strength, maintaining muscle function at a relatively steady level well into the disease

course. In comparison, untreated hSOD1^{G93A} mice experienced steady declines in grip strength throughout the disease course. Though preliminary, these data are incredibly promising for the preclinical efficacy of PCA, and are the first to demonstrate that this anthocyanin metabolite may show potential as a therapeutic agent for ALS. A complete summary of the therapeutic potetial of each treatment tested within the scope of this work can be found in table 6.1.

Table 6.1. Summary of the Neuroprotective, Anti-inflammatory, and Therapeutic Effects of Anthocyanins and Their Metabolites. The *in vitro* neuroprotective and anti-inflammatory effects and the *in vivo* preclinical benefits of treatment with the anthocyanins and anthocyanin metabolites discussed in this dissertation are summarized. + indicates a positive/protective effect and – indicates no effect on the indicated stesses or clinical index of disease that were examined. ND (not determined) indicates that a particular insult or clinical index was not examined for a treatment.

***In Vitro* Effects**				
Treatment	**Oxidative Stress**	**Nitrosative Stress**	**Excitotoxicity**	**Neuro-inflammation**
SAE	ND	-	+	ND
BAE	ND	+	+	ND
Callistephin	ND	-	+	ND
Kuromanin	ND	+	+	ND
HBA	+	-	+	-
PCA	+	+	-	+

***In Vivo* Effects in the hSOD1^{G93A} Mouse**					
Treatment	**Disease Onset**	**Survival**	**Grip Strength**	**Weight**	**Histopathology**
SAE	+	+	+	-	+
PCA	ND	+	+	-	ND

6.2 Considerations for Anthocyanins and Their Metabolites as Therapeutic Agents in ALS

Although the data presented herein are promising with regards to the use of both anthocyanins and their metabolites for ALS, several considerations must be taken into account when choosing a compound for further preclinical and clinical development. These include the relative neuroprotective abilities of these compounds, their availability and absorption in relevant tissues, and the timing and route of treatment administration. Additionally, other considerations beyond targeting neuroprotection in the CNS must also be evaluated when predicting the preclinical and clinical efficacy of these compounds.

6.2.1 Implications of Differential Neuroprotective and Anti-inflammatory Effects of Anthocyanins and Their Metabolites

The anthocyanins kuromanin and callistephin, as well as their metabolites PCA and HBA, are potent antioxidants that are able to defend primary cultures of CGNs from oxidative stress (Kelsey et al. 2011, Chapter 4, section 4.4.1). However, as illustrated in Chapters 2 and 4, subtle variations in the structure of anthocyanins and their metabolites have a significant impact on the chemistry of these compounds, and consequently, their interactions within biological systems under different contexts. Indeed, though these compounds may display similar activity under one condition encountered in the neurodegenerative process, such as oxidative stress, under other disease conditions, their activity may differ substantially from one another. This was most clearly demonstrated *in vitro* under conditions of nitrosative stress induced by nitric oxide.

While both kuromanin and its metabolite, PCA show impressive neuroprotective effects in CGNs insulted with the nitric oxide donor, sodium nitroprusside (SNP), callistephin and HBA offer no protection from nitric oxide toxicity (Chapter 2, Section 2.4.3; Chapter 4, Section 4.4.2). However, when comparing the molecular structures of these compounds, it becomes readily apparent that both kuromanin and PCA possess a catechol moiety, a feature that both callistephin and its metabolite, HBA, lack. In Chapter 2, we define the mechanism of neuroprotection against nitric oxide for kuromanin, and propose that this process is dependent upon auto-oxidation of the catechol moiety on the B-ring of this anthocyanin to generate superoxide. Superoxide then acts in turn as a scavenger of toxic nitric oxide radicals. Since callistephin lacks a catechol structure, it is incapable of participating in this reaction, and offers no neuroprotection. As PCA and HBA can be derived from the B-rings of kuromanin and callistephin respectively, and demonstrate the same neuroprotective capacities as their parent compounds against nitric oxide, it is reasonable to assume that the mechanism of PCA's neuroprotective abilities in this context are similar to those of kuromanin. As nitric oxide toxicity plays a prominent role in the neurodegenerative process, the ability to mitigate nitrosative stress is a desirable feature in any potential therapeutic agent and makes kuromanin and PCA appealing in this regard. However, the complex nature of ALS pathology is also driven by other factors such as excitotoxicity.

In the context of glutamate excitotoxicity, both callistephin and kuromanin acted as effective neuroprotective agents; however the neuroprotective abilities of their metabolites, HBA and PCA, are widely divergent. While HBA offers significant

protection from this insult, PCA does not significantly alter glutamate-induced toxicity (Chapter 4, Section 4.4.3). This again illustrates the significant contribution of chemical structure to differences in the neuroprotective effects of these compounds as HBA and PCA share a significant degree of structural homology, differing only by the presence of an additional hydroxyl group on the phenolic ring of PCA. While the mechanism underlying this effect has not been defined, the ability of anthocyanins and HBA to defend primary neurons from excitotoxic stress represents another desirable therapeutic feature in these compounds. Additionally, the differential neuroprotective abilities of PCA and its parent anthocyanin, kuromanin, highlight another vital consideration for identifying novel therapeutic agents for neurodegeneration in that the beneficial effects of parent compounds are not always retained by their metabolites. This consideration becomes particularly important when assessing the activity of these compounds *in vivo*, where extensive metabolism of parent compounds takes place, particularly with oral consumption.

Lastly, although the anti-inflammatory effects of parent anthocyanins were not evaluated in this work, we again observed substantially different results on the inflammatory phenotype of microglia treated with lipopolysaccharide (LPS) and either HBA or PCA. Indeed, PCA was able to attenuate microglial inflammation as measured through production of nitric oxide and iNOS; however HBA did not display this ability (Chapter 4, Section 4.4.4). While further investigation is needed to define the mechanism by which PCA mitigates iNOS induction and nitric oxide production, this example also highlights how similar compounds can act in very different ways depending on the

context of the disease. Anti-neuroinflammatory activity is yet another desirable trait of potential therapeutic candidates for neurodegeneration given the role of glial inflammation in ALS, making PCA an appealing therapeutic candidate in this regard.

Based upon these considerations, kuromanin and PCA emerge as having the broadest range of neuroprotective activity against diverse sources of neurotoxicity among anthocyanins and their metabolites respectively. This makes them appealing therapeutic candidates for diseases such as ALS; however, while their *in vitro* activity is promising, it is of great import to consider how these compounds may behave *in vivo* before selecting a particular agent for preclinical development.

6.2.2 Bioavailability, Metabolism and Absorption of Anthocyanins and Their Metabolites

As previously discussed, the overall bioavailability of anthocyanins is quite low, particularly in comparison to their phenolic acid metabolites (Youdim et al. 2003, Andres-Lacueva et al. 2005, Passamonti et al. 2005, Talavera et al. 2005, El Mohsen et al. 2006, Vitaglione et al. 2007, Azzini et al. 2010). This is due in part to their extensive degradation in the intestinal tract; however other factors also affect the abundance of these compounds in relevant tissues. It has been described previously that anthocyanins interact in differing degrees with lipid membranes dependent upon the number of hydroxyl groups on the compound (Youdim et al. 2003, Brown and Kelly, 2007, Faria et al. 2014). The capacity to interact with membranes significantly affects the ability of anthocyanins to be absorbed across epithelial and endothelial cell layers *in vitro*, such as

those observed in the intestine and the blood brain barrier (Youdim et al. 2003, Faria et al. 2014). Similar trends have been described for anthocyanin metabolites, which display an increasing ability to mitigate lipid peroxidation in direct correlation with their relative lipophilicity (Brown and Kelly, 2007). This study also demonstrated that phenolic acids like PCA were more effective at interacting with lipid-rich environments and preventing lipid peroxidation than their parent anthocyanins, strongly suggesting that these compounds may be taken up at a higher rate than parent anthocyanins. This data is supported by the observation that PCA accumulates in serum at levels significantly greater than that of parent compounds (Vitaglione et al. 2007, Azzini et al. 2010). However, it is important to note that to our knowledge, there are currently no studies directly evaluating the interaction of phenolic acid metabolites with lipid membranes, and the mechanism by which these compounds are absorbed in the gut after generation by microflora is undefined. Nevertheless, the findings discussed above suggest that parent anthocyanins may not be an ideal choice as therapeutic agents for neurodegeneration, though the relatively high absorption of metabolites like PCA is encouraging.

Metabolism also significantly impacts bioavailability, as observed with anthocyanins, which are degraded in the gut to form phenolic acids and aldehydes in addition to forming methylated, sulfated, glucuronidated, and glycosylated conjugates (Gonthier et al. 2003, Manach et al. 2005, Fleschhut et al. 2006, Woodward et al. 2009, Forester and Waterhouse, 2010, Woodward et al. 2011). Thus, while we observed positive effects on disease onset and lifespan, as well as histopathological indices of disease in the hSOD1^{G93A} mouse model of ALS, it is very likely that these effects were

mediated by anthocyanin metabolites formed *in vivo* after oral ingestion (Chapter 3). Thus, we next explored the therapeutic efficacy of direct supplementation with the anthocyanin metabolite, PCA. PCA administration significantly extended survival in hSOD1^{G93A} mice, and preserved grip strength through the initial portion of the disease course, which provided encouraging results for the therapeutic efficacy of this compound in ALS. However, while the metabolism of anthocyanins has been studied extensively, metabolism of phenolic acids like PCA is less understood. It has been shown *in vitro* that incubation of PCA with liver microsomes forms glucuronidated conjugates of this compound, and a study in healthy human subjects administered cyanidin-*O*-3-glucoside found several PCA-derived metabolites in serum up to several hours after anthocyanin consumption (Woodward et al. 2011, de Ferrars et al. 2014). With this in mind, it will be of great interest in the future to determine how PCA is metabolized when administered directly as a purified substance, as extensive degradation or alterations to this compound could modify its activity and therefore its efficacy as a potential therapeutic for ALS and related diseases.

6.2.3 The "Dying-Back" Phenomenon: Beyond the Neuroprotective Effects of Anthocyanins and Their Metabolites

Since ALS is characterized by the selective death of motor neurons, most therapeutic strategies are designed with the goal of achieving neuroprotection, often measured by preservation of motor neuron cell bodies in spinal cord tissue. However, this assay of neuronal viability does not take into account the functional state of surviving

motor neurons with regards to muscle innervation. Indeed, it has been observed in ALS mouse models that nerve terminals and NMJs begin to degrade early on in the disease while neuronal cell bodies in the spinal cord remain largely intact, leading to the development of the "dying-back" hypothesis (Fischer et al. 2004). This suggests that motor neuron dysfunction begins with loss of axonal health and subsequent axonal retraction, which occurs long before indices of neuronal death are observed in the spinal cord. It is now widely accepted that the dying-back phenomenon precedes overt loss of motor function and neuronal death; however, until recently, many studies to identify novel treatment strategies have focused around physically preserving motor neurons in the spinal cord, but have given little regard to preserving the functionality of these cells for muscle innervation.

Dying-back of motor neuron axons is thought to be the result of multiple intrinsic factors affecting motor neurons such as impaired axonal transport and mitochondrial dysfunction (reviewed by Dadon-Nachum et al. 2011). For example, previous work in our lab has demonstrated that members of the Rho family of GTPases, which play a role in neuronal survival and axonal outgrowth, are dysregulated in spinal cord motor neurons in the hSOD1^{G93A} mouse model of ALS. Indeed, Rac1, which promotes neuronal survival and axonal outgrowth, is diminished in spinal motor neurons of hSOD1^{G93A} mice in comparison to non-transgenic wild type (WT) controls. Conversely, RhoB, which plays a role in promoting neuronal death and axonal retraction, was found to be re-distributed to motor neuron axons in hSOD1^{G93A} mice, while remaining localized in the cell body in WT controls (Stankiewicz, 2014).

Additionally, the role of non-cell autonomous mechanisms has also been implicated in the dying-back phenomenon as new evidence emerges to suggest that skeletal muscle may contribute to axonal dysfunction and subsequent neuronal death (reviewed by Loeffler et al. 2016). Like motor neurons, oxidative stress and mitochondrial dysfunction have been documented in skeletal muscle isolated from mice harboring mutant forms of SOD1. Moreover, several studies have emerged highlighting the beneficial role of targeting skeletal muscle for treatment on overall disease pathology. In particular, one study demonstrated that overexpression of insulin-like growth factor-1 in muscle tissue of hSOD1^{G93A} mice decreased inflammation and motor neuron death in spinal cord tissue in addition to diminishing muscle atrophy and preserving NMJs (Dobrowolny et al. 2005). In a related manner, increases in brain-derived neurotrophic factor (BDNF) in skeletal muscle caused by motor activity correlated with enhanced muscle re-innervation following nerve crush injury, suggestive of a role for muscle-derived neurotrophic factors in muscle innervation (Sartini et al. 2013). Collectively, these data suggest that it is important to examine the effect of novel therapeutic compounds for ALS, such as anthocyanins and their metabolites, on both intrinsic factors and extrinsic muscle-derived factors involved in the retraction of motor axons from skeletal muscle.

While the effects of anthocyanins and their metabolites on the activity and regulation of Rac and Rho have not been examined in neurons, PCA has been shown to modulate the activity of RhoB in melanoma cells (Lin et al. 2011). While PCA treatment increased expression of RhoB in this system, it is important to note that anthocyanins

display opposite effects on cellular signaling pathways in cancer cells and neurons, such as promotion of pro-apoptotic signaling in the former and diminished pro-apoptotic signaling in the latter (Ross et al. 2012). Thus, it is possible that PCA may modulate RhoB expression and activity in neurons in a manner opposite to that observed in melanoma cells. With this in mind, the Rho signaling pathway could represent an interesting point for future examination of therapeutic interventions with PCA to prevent, and perhaps reverse, the dying back phenomenon that is observed to precede motor neuron death in ALS. Furthermore, it has been previously reported that anthocyanins and PCA are capable of increasing BDNF levels in brain through activation of cAMP response element binding protein (Williams et al. 2008, Song et al. 2014) Although it is currently unknown if anthocyanins or their metabolites are also able to modulate BDNF levels in skeletal muscle tissue, this ability could potentially be used to stimulate re-innervation by motor axons and prevent the dying-back phenomenon. This idea is supported by our data demonstrating that anthocyanin supplementation preserves NMJ size and architecture in skeletal muscle tissue from hSOD1^{G93A} mice, which corresponds to preserved muscle function and grip strength (Chapter 3, Section 3.4). As PCA also appears to preserve grip strength in this model, it will be of great interest to evaluate the effects of this compound on NMJs and motor axons (Chapter 5, Section 5.4).

6.2.4 Timing and Standardization of Treatment with Anthocyanins and Their Metabolites

Practical considerations must also be kept in mind when evaluating anthocyanins and their metabolites as potential therapeutic agents for ALS. Chief among these is the time at which these agents can be administered and still provide a measurable effect. Up to this point, many studies have identified potential therapeutic compounds by presymptomatic administration of these substances in ALS mouse models; however the translation of these agents into clinical settings has yielded incredibly poor results on a universal scale (Ludolph et al. 2007, Wilkins et al. 2011). Traditionally, studies have been conducted in this manner to ensure that promising therapeutic candidates are not inadvertently overlooked due to poor performance when administered at later disease stages in animal models displaying very aggressive forms of ALS, such as the $hSOD1^{G93A}$ mouse model (Leitner et al. 2009). Nevertheless, this treatment paradigm is a poor representation of clinical practices. Since the vast majority of ALS patients are diagnosed with the sporadic form of the disease, treatment in the clinic cannot begin until after the initial onset of symptoms. Thus, it is becoming a more common practice to evaluate potential therapeutic candidates at both presymptomatic ages and the time of disease onset, which represents a more clinically relevant time point for translational research.

In the present work, we defined the therapeutic benefits of administering anthocyanins at a presymptomatic age and observed a significant shift in disease progression as well as positive impacts on grip strength and histopathological indices of

disease (Chapter 3). However, while these results are very encouraging, it is important to note that the poor correlation between the success of potential therapeutic agents in preclinical animal models and the success of these compounds in human patients necessitates evaluation of the therapeutic efficacy of anthocyanins at a later disease stage. However, given that the beneficial effects of anthocyanin treatment in the hSOD1^{G93A} mouse model of ALS were likely mediated by anthocyanin metabolites rather than the parent compounds, we instead evaluated the therapeutic benefits of the anthocyanin metabolite, PCA, at disease onset. Preliminary data in this study demonstrated that PCA significantly slows disease progression in treated mice when administered at this later disease stage, providing further compelling evidence that PCA may be a viable therapeutic candidate for the treatment of ALS, particularly in light of its success at a clinically relevant time point in the disease.

One more practical consideration for the use of anthocyanins and their metabolites for the treatment of ALS is the ability to standardize the production and use of these compounds. Currently, purified anthocyanins are very costly, one reason many investigators choose to evaluate the beneficial effects of these compounds using anthocyanin-enriched extracts. However, the success of anthocyanin extraction is dependent upon many factors such as temperature, solvent, and pH, which can significantly affect the anthocyanin content of the end product (Revilla et al. 1998, Lu and Foo, 2001, Kahkonen et al. 2003, Lapornik et al. 2005, Monrad et al. 2010). Furthermore, solvents that are safe for human consumption following extraction, such as ethanol and water, are less efficient for extracting anthocyanins from plant material than

solvents such as methanol that carry a risk of toxicity (Lapornik et al. 2005). Thus, the use of anthocyanin extracts on a large scale for the treatment of diseases such as ALS poses many challenges with regards to standardization and quality control, while the use of commercially available purified anthocyanins is cost prohibitive. Phenolic acids such as PCA, however, are commercially available as highly purified isolates for a relatively low cost, making this compound highly appealing for drug development and future clinical use. Thus, with these and the aforementioned considerations in mind, we conclude that PCA is the most promising therapeutic candidate of the compounds examined in this work, and suggest that this compound may be of significant therapeutic benefit for the treatment of ALS. Nevertheless, further study is required to more rigorously examine the effects of PCA administration on disease progression and muscle function, as well as to define its mechanisms of action *in vivo* in the preclinical hSOD1^{G93A} mouse model.

6.3 Future Directions for Therapeutic Development

In the future, it will be of considerable interest to assess the effects of PCA supplementation on histopathological indices of disease in order to determine its mechanism of action. Given the very promising effects PCA administration has had on clinical indices of disease, such as survival and hind limb grip strength, we predict that we will observe positive effects on ALS disease pathology. Taken together with the considerations discussed above, we suggest that PCA may be of significant clinical

benefit to patients suffering from this devastating disease, and following further preclinical assessment, we hope to pursue a clinical trial with this compound.

Additionally, it would be of significant clinical relevance to evaluate the potential additive or synergistic effects with other established and experimental treatments for ALS. For example, *in vitro* experiments demonstrated that PCA does not protect neurons from glutamate excitotoxicity. Therefore, combining this compound with Riluzole, an anti-glutamatergic drug, could have significant synergistic effects on ALS disease progression and pathology as it would target a broader array of factors contributing to motor neuron death.

PCA may also be uniquely suited for administration in studies evaluating the efficacy of stem cell therapy for ALS. While these studies initially sought to replace dying motor neurons with those derived from healthy stem cells, this approach had many obstacles, and current research has now turned to protecting existing motor neurons by implanting neural progenitors into spinal cord tissue, which then differentiate into healthy, wild type astrocytes (reviewed by Haidet-Phillips and Maragakis, 2015). Astrocytes derived from this process are then thought to integrate into spinal cord tissue and secrete protective neurotrophic factors to support and repair injured motor neurons. This strategy has yielded promising results in rodent models of ALS, and recently, a Phase I clinical trial assessing the safety and feasibility of stem cell injection into the spinal cord of ALS patients was completed (Lepore et al. 2008, Glass et al. 2012, Feldman et al. 2014, Kondo et al. 2014, Chen et al. 2015). Concerns still exist, however, as to relative survival and differentiation of transplanted stem cells, and speculation as to

whether stem cell-derived astrocytes will become inflammatory upon encountering the toxic environment of the ALS spinal cord as arisen (Haidet-Phillips and Maragakis, 2015). Recently, however, it was shown that PCA is able to enhance differentiation of rat neural progenitors into neurons *in vitro* in addition to decreasing cell death in differentiating cell populations of both neurons and glia (Guan et al. 2009, Guan et al. 2011). While PCA modestly decreased the number of cells that differentiated into astrocytes in this paradigm, these studies also reported increases in antioxidant enzymes and decreased levels of reactive oxygen species in all differentiated cell types in the presence of PCA (Guan et al. 2011). This suggests that the overall health of the astrocytes derived from this process may be superior to that of astrocytes derived under standard differentiating conditions, which could prevent their transition to an inflammatory state. Taken together with our data demonstrating that PCA may be an effective anti-inflammatory compound, this suggests that PCA administered in tandem with neural progenitors in ALS spinal cord may encourage stem cell differentiation and survival in addition to preventing astroglial inflammation once new astrocyte populations are established.

6.4 Conclusions

In conclusion, this dissertation has established the therapeutic potential of two anthocyanins, callistephin and kuromanin, and their metabolites, HBA and PCA, for the treatment of ALS. Specifically, our work has defined the differential neuroprotective and anti-inflammatory capabilities of these compounds *in vitro*. In addition, this work is the

first to evaluate the use of an anthocyanin-enriched extract, and the anthocyanin metabolite, PCA, as therapeutic agents in the preclinical hSOD1^{G93A} mutant mouse model of ALS. The results of this work provide important data highlighting the therapeutic efficacy of these compounds *in vivo*, and suggest that the use of anthocyanins and their metabolites as therapeutic agents warrants further investigation and consideration for future clinical applications.

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APPENDIX A: ABBREVIATIONS

6-OHDA: 6-hydroxydopamine
AIF: Apoptosis-inducing factor
ALS: amyotrophic lateral sclerosis
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA: analysis of variance
APAF-1: apoptosis protease activating factor-1
APP: amyloid precursor protein
ASK1: apoptosis signal-regulating kinase 1
BAE - blackberry anthocyanin extract
BBB: blood brain barrier
BDNF: brain derived neurotrophic factor
BiP: binding immunoglobulin protein
BSA: bovine serum albumin
BTx: α-bungarotoxin
CGN: cerebellar granule neurons
CHOP: C/EPB homologous protein
CNS: central nervous system
COX-2: cyclooxygenase-2
CytC: cytochrome c
DMEM: Dulbecco's Modified Eagle Medium
DMSO: dimethyl sulfoxide
DPPH: 1,1-diphenyl-2-picrylhydrazyl
EAAT2: excitatory amino acid transporter-2
EGFP: enhanced green fluorescent protein
ER: endoplasmic reticulum
ERK1/2: extracellular signal-regulated kinase 1/2
FasL: Fas ligand
FBS: fetal bovine serum
FUS: fused in sarcoma
GA: gallic acid
γ-GCL: γ-glutamylcysteine ligase
GFAP: glial fibrillary acidic protein
GSH: glutathione
GSSG: oxidized glutathione
H_2O_2: hydrogen peroxide
HBA: 4-hydroxybenzoic acid
$HO2^{\bullet}$: superoxide radical
HRP: horseradish peroxidase
$hSOD1^{G93A}$: human Cu, Zn-superoxide dismutase with a glycine to alanine substitution at position 93
IACUC: institutional animal care and use committee
IL: interleukin

iNOS: inducible nitric oxide synthase
JNK: c-Jun-N-terminal kinase
LPS: lipopolysaccharide
MCLA: 2-Methyl-6-(4-Methoxyphenyl) -3,7-Dihydroimidazo[1, 2-A]pyrazin-O-3ne, Hydrochloride
MCP-1: monocyte chemoattractant protein-1
MPP+: 1-methyl-4-phenylpyridinium
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR: mammalian target of rapamycin
NF-κB: nuclear factor-κB
NMDA: N-methyl-D-aspartate
NMJ: neuromuscular junction
NO: nitric oxide
NOX-2: NADPH-oxidase-2
Nrf2 : nuclear factor erythroid 2-related factor 2
OCT: optimal cutting temperature
$ONOO^-$: peroxynitrite
ORAC: oxygen radical absorption capacity
p38-MAPK: p38-mitogen activated protein kinase
PaGE: paw grip endurance
PBS: phosphate buffered saline
PCA: protocatechuic acid
PDI: protein disulfide isomerase
PEG-SOD: polyethyleneglycol-superoxide dismutase 1
PERK: endoplasmic reticulum kinase
PI3K: phosphoinositide-3-kinase
PS1: presenilin-1
PTEN: phosphatase and tensin homolog
PVDF: polyvinylidene difluoride
QR1: quinone oxidoreductase 1
QR2: quinone oxidoreductase 2
RNS: reactive nitrogen species
ROS: reactive oxygen species
SAE: strawberry anthocyanin extract
SAMP8: senescence-accelerated mouse prone 8
SEM: standard Error of the Mean
SNP: sodium nitroprusside
SOD1: Cu, Zn-superoxide dismutase
SOD2: Mn-superoxide dismutase
TDP-43: TAR DNA-binding protein-43
TFA: trifluoroacetic acid
TNF-α: tumor necrosis factor-α
UPR: unfolded protein response
WT: wild type

APPENDIX B: LIST OF PUBLICATIONS

Chapter 2, entitled “Chemical Basis for the Disparate Neuroprotective Effects of the Anthocyanins, Callistephin and Kuromanin, Against Nitrosative Stress” is in revision for the Journal of Biological Chemistry.

Chapter 3, entitled “An anthocyanin-enriched extract from strawberries delays disease onset and extends survival in the hSOD1^{G93A} mouse model of amyotrophic lateral sclerosis” is in preparation for submission to the Journal of Neuroscience.

Chapter 4, entitled “Comparison of the distinct neuroprotective and Anti-Inflammatory effects of the phenolic acids, protocatechuic acid and 4-hydroxybenzoic acid” is in preparation for submission to the Journal of Neurochemistry.